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RETROVIRAL VECTOR FOR THE TRANSFER AND EXPRESSION OF GENES FOR THERAPEUTICAL PURPOSES IN EUCARYOTIC CELLS

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The invention is based on work performed starting from a particularly virulent strain of the Friend virus. The isolate I-5 of the ecotropic Friend murine leukemia virus was obtained from long-term bone marrow cultures infected by the Friend virus complex which induces polycythemia (FV-P) (Mathieu-Mahul et al., 1982). The FB29 strain of F-MuLV derived from the isolate I-5 (Sitbon et al., 1986) is responsible for cytolytic and leukemogenic effects on erythroid cells, leading to severe early hemolytic anemia followed by late erythroleukemia in susceptible mice inoculated at birth.

The inventors were interested in the specific properties of this strain FB29 and have used it to define retroviral vectors.

Claim

1.   Recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence inserted in said vector characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence given in Figure 1A and nucleotide 1527 of the sequence given in Figure 1C, said control fragment having a 5' LTR sequence included between nucleotides 7842 and 144, a PBS site starting at

nucleotide 145 of the sequence given in Figure 1A, and a packaging sequence included in the sequence of 250 nucleotides following the end of the LTR sequence, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

2.   Recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence inserted in said vector characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence in Figure 1A and nucleotide 310 of the sequence shown in Figure 1D said control sequence having a 5' LTR sequence included between nucleotides 7842 and 144 of Figure 1A, a PBS site starting at nucleotide 145 of Figure 1A, a packaging sequence comprised in the sequence of 250 nucleotides following the end of the 5' LTR sequence, and a 3' LTR sequence included between nucleotide 7842 of the sequence given in Figure 1C and nucleotide 144 of the sequence given in Figure 1D, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

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(54) Title: RETROVIRAL VECTOR FOR THE TRANSFER AND EXPRESSION OF GENES FOR THERAPEUTICAL PURPOSES IN EUCARYOTIC CELLS

(54) Titre: VECTEUR RETROVIRAL POUR LE TRANSFERT ET L'EXPRESSION DE GENES A VISEE THERAPEUTIQUE, DANS DES CELLULES EUCARYOTES

(57) Abstract

The invention relates to a recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence, said vector being characterized in that it comprises any sequence contained in the fragment C-lal-PvuII comprising the nucleotides 7702 and 1527 of the sequence given in figure (1) and comprising the chain LTR comprised between the nucleotides 7842 and 144, the site PBS starting at the nucleotide 145, the encapsidation sequence comprised in the chain of 250 nucleotides following the end of the LTR sequence, said sequence being capable of controlling the cloning and/or expression and/or tranfer of the exogenous sequence, regardless of its transcriptional orientation with respect to the transcriptional orientation of the virus. The invention also relates to the utilisation of said vector for the transfer and/or cloning and/or expression of genes particularly for a genic therapy.

(57) Abrégé

L'invention concerne un vecteur recombinant pour le clonage et/ou l'expression et/ou le transfert d'une séquence de nucléotides exogène caractérisé en ce qu'il comprend toute séquence contenue dans le fragment clal-PvuII comprenant les nucléotides 7702 et 1527 de la séquence donnée à la figure 1 et comprenant l'enchaînement LTR compris entre les nucléotides 7842 et 144, le site PBS débutant au nucléotide 145, la séquence d'encapsidation comprise dans l'enchaînement de 250 nucléotides suivant la fin de la séquence LTR, ladite séquence étant capable de contrôler le clonage et/ou l'expression et/ou le transfert de la séquence exogène, quelle que soit son orientation transcriptionnelle par rapport à l'orientation transcriptionnelle du virus. Elle est relative à l'utilisation de ce vecteur pour le transfert et/ou le clonage et/ou l'expression de gènes notamment dans le cadre de la théraphie génique.

RETROVIRAL VECTOR FOR THE TRANSFER AND EXPRESSION OF GENES FOR THERAPEUTIC PURPOSES IN EUKARYOTIC CELLS

The object of the invention is novel retroviral vectors, particularly for the transfer and expression of genes in eukaryotic cells. In this respect the invention proposes vectors particularly suitable for use in the transfer of genes for clinical therapeutic, prophylactic or diagnostic purposes.

The rapid development of molecular genetics has led to the identification of an increasing number of molecular abnormalities responsible for human diseases. Within the function unit constituted by the gene regions responsible for the expression of a biological signal and its regulation lie side by side. Each of these regions is liable to be the seat of pathological changes leading to a qualitative or quantitative abnormality of synthesis. The detection of these abnormalities allows screening for them but the major objective remains therapeutic.

The transfer of genes for therapeutic purposes or somatic "gene therapy" consists of inserting a "repairer" gene in the somatic cells of a constituted organism in order to compensate for the dysfunction of an endogenous gene, or even to add a novel function for a therapeutic purpose. The resulting genetic change is likely to be transmitted to the daughter cells of the manipulated cell but it will not be inherited. The normal counterpart of impaired DNA sequences is thus transformed into a medicine.

The field of gene therapy is today being very actively developed and combines clinical assays (for still very small patient populations) with very fundamental research work in to matters such as the modes of gene expression or the vectorization of the therapeutic nucleic acid sequences. The vectors presently used are derived either from inactivated viruses, such as retroviruses or adenoviruses, or macromolecular complexes. The retroviruses are more suitable for use in a target tissue comprising a contingent of stem cells capable of being manipulated ex vivo; on the other hand, when the target tissue is constituted of terminally differentiated cells or intimately enmeshed in an organ whose architectural constraints have major functional consequences, such as the lung,

the transfer of genes must be performed <u>in vivo</u> for example by means of adenoviruses. Gene therapy finds applications in diseases as diverse as hereditary diseases due to the alteration of a single gene, such as Duchenne's myopathy, lysosomal diseases, mucoviscidosis or acquired diseases such as AIDS, cancers, thrombo-embolic disease or degenerative neurological diseases, constitutional hematological diseases.

Nonetheless, although the potential applications of gene transfer are extraordinarily large, the therapeutic developments of this approach and its appropriateness still come up against technological difficulties.

In this connection, the development of retroviral vectors more efficacious than the existing tools constitute a major objective. In fact, the retroviral vectors have demonstrated their efficacy in systems in which the target cells of the transfer are classically the subject of mitoses and ideally involve a contingent of stem cells; but the limitations are linked essentially to inadequate infectivity of the viruses used and/or a too moderate level of transcription. For this purpose useful vectors may be selected by considering in particular their infective titer.

The object of the invention is to propose more efficient vectors than those existing, most of which are presently derived from the backbone of the Moloney murine leukemia virus.

The invention is based on work performed starting from a particularly virulent strain of the Friend virus. The isolate I-5 of the ecotropic Friend murine leukemia virus was obtained from long-term bone marrow cultures infected by the Friend virus complex which induces polycythemia (FV-P) (Mathieu-Mahul et al., 1982). The FB29 strain of F-MuLV derived from the isolate I-5 (Sitbon et al., 1986) is responsible for cytolytic and leukemogenic effects on erythroid cells, leading to severe early hemolytic anemia followed by late erythroleukemia in susceptible mice inoculated at birth. The regions responsible for the erythroleukemia were localized in the U3 region of the viral LTR (Sitbon et al., 1986; Sitbon et al., 1991). The principal determinant of the hemolytic anemia seems to depend on specific envelope sequences of the FB29 strain; its severity may be affected by three distinct regions, including a structural segment of the envelope, enhancer sequences of transcription localized in the

U3 region and, finally, sequences of the U5-gag-pol regions (Sitbon et al., 1990).

Furthermore, electron microscopical analyses of the viral particles have confirmed a significantly higher packaging capacity (1.5 to 2 log).

The inventors were interested in the specific properties of this strain FB29 and have used it to define retroviral vectors.

According to a first embodiment, the object of the invention is a recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence inserted in said vector characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence given in Figure 1A and nucleotide 1527 of the sequence given in Figure 1C, said control fragment having a 5' LTR sequence included between nucleotides 7842 and 144, a PBS site starting at nucleotide 145 of the sequence given in Figure 1A, and a packaging sequence included in the sequence of 250 nucleotides following the end of the LTR sequence, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

According to another embodiment of the invention, the recombinant vector is characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence in Figure 1A and nucleotide 310 of the sequence shown in Figure 1D said control sequence having a 5' LTR sequence included between nucleotides 7842 and 144 of Figure 1A, a PBS site starting at nucleotide 145 of Figure 1A, a packaging sequence comprised in the sequence of 250 nucleotides following the end of the 5' LTR sequence, and a 3' LTR sequence included between nucleotide 7842 of the sequence given in Figure 1C and nucleotide 144 of the sequence given in Figure 1D, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

It will be appreciated that according to the first embodiment,

said fragment corresponds to a Cla I-PvuII fragment of FB29 strain virus, and according to the second embodiment, said fragment corresponds to a Cla I-BamH1 fragment of FB29 strain virus.

The abovementioned ClaI, PvuII and BamHI sites originated in the FB29 strain virus. The BamH1 site refers to that which is located at approximately nucleotide 310 of Figure 1D.

It should be noted that during construction of of the retroviral vectors or vectors destined for the production of packaging lines, these sites may be modified and in particular replaced, optionally creating distinct enzymatic cleavage sites.

As is readily seen in Figure 1, the LTR sequence is duplicated, such that the 5' LTR is situated approximately between nucleotide 7842 and nucleotide 144 of the sequence shown in Figure 1A, and the 3' LTR is situated approximately between nucleotide 7842 of the sequence shown in Figure 1C, and nucleotide 144 of the sequence shown in Figure 1D.

A vector comprising the ClaI-PvuII fragment, such as according to the first embodiment, therefore includes the 5' LTR.

A vector comprising the ClaI-BamHI fragment such as according to the second embodiment, thus contains two LTR sequences, 5' LTR and 3' LTR having the same viral origin. This vector may be modified by deletion of all or part of the viral envelope sequence present upstream and/or downstream from the sequences 5' LTR and 3' LTR. A vector of this type is for example pFOCH29-PL described in Figure 7.

These LTR sequences may be separated in the vector by the presence of the gag sequence referred to above and/or by the exogenous nucleotide sequence which it is desired to transfer, clone or express.

According to an attractive embodiment of the invention, the recombinant vector is characterized in that it consists of the fragment situated between nucleotide 7702 of the sequence shown in Figure 1A, and nucleotide 1527 of the sequence shown in Figure 1C.

Another preferred retroviral vector consists of the fragment situated between nucleotide 7702 of the sequence shown in Figure 1A and nucleotide 310 of the sequence shown in Figure 1D.

A retroviral vector of the invention can be used for therapeutic

or diagnostic purposes in order to introduce into the patient a nucleotide sequence of clinical importance. The vector of the invention in fact exhibits the properties of efficiency and safety required for this application

5   Advantageously, the control of cloning, expression or transfer of the exogenous sequence is achieved according to the invention, irrespective of the transcriptional orientation of this sequence with respect to the transcriptional orientation of the virus.

An exogenous nucleotide sequence according to the invention

10   is a nucleotide sequence which is not naturally contained in the genetic material constituting the vector and, in particular, in the sequences necessary for the control of the expression, cloning or transfer. It may be a natural or synthetic sequence, in particular a hybrid sequence.

By the expression "transfer of an exogenous nucleotide sequence" is meant the incorporation of a sequence borne by the vector into the genome or satellite of this latter of a cell transformed by this vector. Such a transfer may be the result of recombination, in particular homologous recombination.

The vector of the inventim may thus allow the pemnanent expression in the genome of a target cell of a sequence of exogenous nucleotides selected for its property of integration into the genome of the target cells.

According to an attractive embodiment of the invention, the exogenous sequence and the sequence contained in the ClaI-PvuII fragment or in the ClaI-BamHI fragment or in one of these fragments according to the above description are inserted in a plasmid, for example in the plasmid Puc19, plasmid Pucl8 or any other suitable plasmid.

Preferably the recombinant vector additionally comprises a part of the gag sequence situated between the nucleotides 619 and 2245 of the sequence shown in Figure 5, in particular the sequence included between nucleotides 619 and 1527 of the sequence shown in Figure 5.

The presence of a fragment or all of the gag sequence may contribute to the stabilization of the vector obtained. The gag sequence codes for the nucleoproteins of the Friend virus and increases the packaging efficiency, at least in its proximal part.

However, it may be useful to limit the part of the gag sequence present in the vector of the invention as a function of the size of the exogenous sequence introduced into the vector in order to obtain a higher infective viral titer and to diminish the production of viral proteins and the risk of generating replication-competent viruses. Preferably, the part of the gag sequence present in the vector should be less than about 2/3 of the normal gag sequence. Advantageously, the conserved gag sequence is the part of this sequence implicated in the packaging step of the retroviral vector obtained.

A useful vector of the invention is characterized in that it essentially lacks the viral sequences pol and/or env.

On the other hand, the pol and gag sequences of the FB29 strain as shown in Figure 5 may be conserved downstream from the

LTR for the production of packaging lines (see Figure 23 as an example).

The expression of the pol and gag sequences can be controlled by a promoter distinct from the viral LTR which is then deleted.

5   The object of the invention is thus constructions such as described above comprising said control sequence contained in the ClaI-PvuII fragment previously mentioned and comprising in addition the gag and pol sequences or a part of these sequences sufficient for the production of packaging lines.

10   These lines may be used to package the retroviral vector of the invention.

A vector comprising both the ClaI-PvuII fragment (which bears a unique LTR sequence) and the gag and pol sequences or a part of these sequences may in particular be used in the context of

15   the preparation of packaging lines for ex vivo or in vivo gene transfers, the said gene being represented by the exogenous nucleotide sequence

When a part of the env viral sequence is present in the vector it remains insufficient to allow recombinations likely to produce

20   replication-competent viruses.

A useful vector of the invention lacking the envelope sequence consists of the fragment comprising nucleotides 7806 to 1527 of the sequence shown in Figure 1A, B and C.

The invention also relates to a recombinant vector

25   characterized in that said control sequence contained in said fragment is replaced by a sequence hybridizing under conditions of high stringency with said control sequence or is replaced by a sequence having a nucleotide homology of at least 95% with said control sequence or of at least 85% with the control sequence.

30   Hybridization is performed in the same hybridization media as those described in the experimental part by adding, however, two rinsings for 10 min at 65 C in a 1x SSC, 0.1 SDS medium as well as 2 rinsings for 10 min at 65 C in a 0.1x SSC, 0.1 SDS medium.

The nomenclature of the nucleotides is given above by reference to the numbering of the nucleotides of the viral sequence shown in Figure 1.

Optionally, one of the two LTR sequences previously defined starting from the sequence of the F-MuLV virus (strain FB29 of the Friend virus) may be replaced by a LTR sequence derived from another virus, for example from the Moloney murine leukemia virus (Mo-MuLV).

Similarly, the recombinant vector of the invention may also contain other retroviral sequences than those which have been described above, either derived from the same F-MuLV virus whose sequence is given in Figure 5, or derived from another virus.

The object of the invention is also a recombinant vector characterized in that it contains in addition at least one polylinker possessing uniques restriction sites with respect to the sites contained in the vector.

Such an adaptor, preferably with multiple sites (polylinker), permits in particular the insertion of one or more exogenous sequences whose transfer, cloning or expression is desired.

A perticularly useful vector is the vector characterized in that it is the plasmid pFOCH29 deposited under the designation pFOCH29-SCS1 in a strain of E. coli SCS1 with the C.N.C.M.on 30 June 1993 under No. I-1326.

The strain E. coli SCS1 is marketed by the STRATAGENE Corp company.

A vector of the invention may also contain a marker gene or part of a marker gene such as for example the gene for neomycin resistance. The presence of a marker gene facilitates in particular the detection of the presence of the vector in recombinant cells.

The object of the invention is also a recombinant vector complying with the foregoing specifications in which the U3 region of the LTR is deleted at least in part such that the transcriptional sequences in particular the promoter and/or enhancer contained in U3 is (are) at least in part inactivated or modified.

In this case, the vector is capable of autoactivation or is a SIN vector ("self inactivating vector"). A SIN vector thus constructed allows the expression of the exogenous sequence which it contains when the latter is placed under the control of a so-called "internal

promoter", viral or non-viral in nature, optionally the promoter of this sequence, or a promoter such as the promoter of the EGF (epidermal growth factor) receptor or the ubiquitous PGK promoter of phosphoglycerate kinase.

The advantage consists in improving safety (non propagation and diminution of the risk of activation of neighbouring sequences). Another advantage is to profit from the integration mediated by the retroviruses but to specify or target transcription by the internal promoter.

Similarly, the U5 sequence, even if necessary the sequence R, may be deleted at least in part.

This deletion may be performed at the unique LTR sequence present in the vector or optionally at each LTR sequence of this vector.

However, this leads most often to a diminution of the viral particle titer, even to the absence of integration into the genome of the target cell (if U5 deleted).

According to another embodiment of the invention, the exogenous nucleotide sequence is under the control of an exogenous (or internal) promoter.

By "exogenous promoter" is meant a promoter which is not naturally present in the vector. Such a promoter may be the natural promoter of the exogenous sequence. It may be a constitutive promoter or an inducible promoter.

A previously defined recombinant vector is preferably introduced into a packaging line for example by transfection or electroporation. This transfection allows the constitution of viral particles intrinsic to the production of recombinations by transduction in target cells for the purpose of cloning, transfer or expression of the exogenous nucleotide sequence contained in the vector.

Thus, a particularly useful vector may be transfected into the psi-CRIP line.

It is also possible to have recourse to the packaging line psi-CRE or to any other line provided that it does not lead to recombinations likely to give rise to the production of wildtype viral particles from the proviral DNA contained in the vector.

According to another embodiment of the invention, the recombinant vector may be introduced into liposomes or into a macromolecular complex (Monsigny M et al. M/S 1993).

The F-MuMV vector may be used to produce such packaging lines according to the procedure illustrated in McLachlin JR et al., 1990. Such a line may be constructed from sequences of the gag, env and pol genes, the packaging sequence being deleted and at least one of the gag, pol or env sequences bearing a point mutation which does not adversely affect the resulting protein.

The vectors of the invention may contain one or more exogenous sequences. These sequences may be inserted outside the ClaI-BamHI or BamHI-BamHI fragments as was seen above or, on the contrary, may be inserted within these fragments and in particular in their LTR sequence.

These vectors may also contain cell targeting elements to orient the integration of the vector in specific cells.

Advantageously, on infection of the target cells, the retroviral vector constructions according to the invention lead to a viral titer equal to or higher than 10^4 pfu/ml evaluated when the exogenous nucleotide sequence coding for the neomycin gene is inserted into this vector.

This vector may have very diverse uses and in particular these vectors may be used for the cloning, expression and/or transfer of nucleotide sequences having clinical (therapeutic or diagnostic) importance.

Thus it is possible to use the vectors of the invention for the transfer into cells, for example somatic cells, of genes for therapeutic purposes whatever the disorder(s) or disease(s) concerned.

The therapeutically important sequences referred to here are for example sequences corresponding to the normal equivalent of a non-functional gene in the case of a given disease or also of an antisense sequence or a dominant negative mutant of a given gene or a sequence coding for a functional inhibitor of a gene or the use of a marker gene.

The vector of the invention is thus appropriate for the gene therapy of cancer by application of gene correction techniques or improvement of the strategies for the destruction of tumor cells.

correct constitutional mutations in the case of hereditary predisposition to cancer, abnormalities of the signal transduction like the pathways mediated by the ras oncogene and its homologues, enhancer abnormalities of oncogenes, inhibitory abnormalities of tumor suppressor genes, abnormalities promoting genetic instability, abnormalities affecting DNA repair.

According to the second approach the vector may be used to activate prodrugs as in the case of the thymidine kinase gene of the herpes virus which converts Gancidovir or Acyclovir into cytotoxic drugs, or the cytosine deaminase gene which converts a precursor of 5-fluorouracil into an active drug, or to induce or stimulate the immune system by manipulation of tumor cells with for example cytokine genes, by manipulation of antigen-presenting cells or their precursors (hematopoietic stem cells) or by manipulation of the immunity effector cells, T cells, B cells, LAK, TILs.

When the correction of genetic diseases and anemias is involved, the invention can for example be applied to the correction of inborn errors of metabolism, hemoglobin diseases such as thalassemias or sickle cell anemia, diseases of hemostasis and coagulation, hereditary diseases of demyelination or myopathies.

The vectors of the invention are also suitable for vaccinating patients against pathological agents, either permanently or transiently.

The transfer may be achived by transduction into cells, tissues, organs or organisms.

According to another embodiment of the invention, the exogenous nucleotide sequence codes for anantigen or an antigenic determinant.

A vector containing such an antigenic determinant should be used as permanent or transient vaccine or optionally in the context of a therapeutic protocol for example to provoke an immune response.

As an example, sequences of HIV retroviral antigens may be incorporated into the vector of the invention.

In this connection, the retroviral vector of the invention may be used for intracellular immunization by using transdominant mutants of CD4, Tat, Rev, Gp120, decoys with excessive synthesis of

regulatory proteins such as TAR, specific ribozymes of viral sequences or antisense genes.

This same procedure may be used to treat other retroviral infections.

The exogenous nucleotide sequence previously mentioned may be a sequence of genomic DNA or a cDNA sequence or also an RNA sequence.

Similarly, this sequence may be natural or synthetic.

The object of the invention is also a eukaryotic or prokaryotic recombinant cell characterized in that it is modified by a recombinant vector of the invention. Advantageously, it is a cell of a species not bearing an endogenous retrovirus.

Such a cell is advantageously a mammalian cell, in particular a human cell.

Similarly, it is either a totally differentiated cell or a cell of the precursor type. For example, the vector of the invention is particularly suitable for the modification of hematopoietic cells and also hematopoietic cell precursors or of a cell of a lymphomyeloid totipotent strain.

Moreover, glial or neuronal nerve cells may also be modified by the vector of the invention.

Other cells may be modified by the vectors of the invention for example T or B lymphocytes, or other mediators of cell immunity, tumor cells, medullary stroma, endothelial or mesenchymatous cells.

The recombinant vector according to the invention may also be used to modify fibroblasts, cutaneous cells, hepatic cells or muscle cells.

Other cellular targets may be transformed by the vector of the invention. Mention should be made of epithelial cells, for example of the mammary or vesicular epithelium, tumor cells, accessory cells of the nervous system such as precursors of oligodendrocytes or Schwann cells.

It is also possible to modify cell lines such as lines of JURKATT T lymphocytes, NK cell lines such as YT2C2, lines of monocytes-macrophages (for example U937) or erythro-megakaryocyte lines (for example K562).

Other characteristics and advantages of the invention will become apparent in the examples and Figures which follow.

Figure 1 : Sequence of viral DNA used for the construction of the vector FOCH29.

Figure 2 : A : Restriction map of the vector FOCH29.
References Seq "FB29"

Cla   ----> U3 140 (7702-7845)

U3   ----> R 410 (7845-8255)

R   ---> CBS 145 (0-145)

PvuII   -----> BamHI 208
PvuII   ----> PvuII 1098
PvuIIMT   ----> PvuII 1669
BsmAI   ----> 55/150/765/1766/2531/2684

B : Restriction map of FOCH 29 in the which the sites are indicated using the numbering of the FB 29 sequence shown in figure 1 and with the numbering intrinsic to the construction thus achieved (numbers in parentheses).

Figure 3 : Restriction map of the vector FOCH29-Neo. The neo gene was cloned into the polylinker. The enzymes which can be used in the polylinker are Xba, Sal, SphI
Cuts : - BglII 2070
              5300
         - Bam 1392
              1500
              4456
         - Sal/BglII   300
                   2070
                   5000

Figure 4 : Restriction map of the plasmid pUC19

Figure 5 : Sequence of F-MuLV

Figures 6 to 24 : Restriction maps of the constructions produced from the retroviral vector FOCH29

Examples

A) PREPARATION OF THE RETROVIRAL VECTOR

MATERIALS AND METHODS

1 - Source of the viral genomic material

The genomic DNA of the provirus was cloned in pBR322 (Sitbon et al., 1986). After replacement of the ClaI site at 7702 of the viral sequence by a EcoRI site, the EcoRI-PvuII fragment of 2110 base pairs (bp) containing all of the viral Long Terminal Repeat (LTR) was subcloned at the EcoRI and SmaI sites of the polylinker of pUC19.

2 - Construction of the retroviral vector FOCH29

The HindIII site of the polylinker of pUC19 containing the EcoRI-PvuII fragment was replaced by a BglII site, after opening by HindIII, filling in with the long fragment of E. coli DNA polymerase (Klenow fragment) and ligation with a BglII adaptor not recreating the HindIII site; the BglII site was introduced to receive a BamHI-BamHI fragment of 865 bp containing a second copy of the native LTR of the Friend virus destined to constitute the downstream LTR (or 3' LTR). This BamHI-BamHI fragment was isolated by replacing the EcoRI site of pUC19 by a BamHI site upstream by means of a linker not recreating the EcoRI site after filling in of the ends by the Klenow fragment of the DNA polymerase; the BamHI site downstream is endogenous to the viral sequence.

This fragment was thus introduced by ligation with the backbone (pUC19) whose opening by BglII made it possible to combine the cohesive ends with the ends generated by BamHI. The resulting plasmid is called pFOCH29.

3 - Introduction of a marker gene

The BglII-BamHI cDNA fragment (1500 bp) of the gene for neomycin resistance derived from the retrotransposon Tn5 (NeoR) was introduced between the two viral LTRs after ligation of the three fragments : pUC19-5' LTR BglII, NeoR BglII-BamHI and 3' LTR BamHI-BamHI. The resulting plasmid is called pFOCH29-Neo.

4 - Transfection of packaging lines psi-CRIP and infection of fibroblasts

The plasmid pFOCH29-Neo was introduced into the amphotropic packaging line psi-CRIP described by Danos et al. (1988) by transfection using calcium phosphate precipitation according to the standard procedure without DNA carrier; 10 micrograms of plasmid were deposited on a culture dish 35 mm in diameter on which 5×10^4 cells were seeded the day before.

The psi-Crip cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% newborn calf serum (Hyclone). Two days after transfection the cells were trypsinized, diluted 1/20 and subjected to selection in the presence of geneticin at a final concentration of 1 milligram (mg) per milliliter (ml) of culture medium. The colonies which appeared after 12 days were selected and reimplanted on 24-well culture dishes at a concentration of one clone per well.

The cell culture supernatant of a well which had reached confluence was taken, filtered through a 0.45 μm filter to remove cells in suspension and used to infect mouse fibroblasts (NIH3T3) seeded identically on 24-well culture plates in the presence of polybrene at a concentration of 8 μg/ml. The NIH3T3 were grown in DMEM supplemented with 10% fetal calf serum (FCS). Viral integration was analysed by PCR on a lysate of NIH3T3 which had reached confluence.

5 - Polymerase chain reaction (PCR)

The lysate supernatant of the confluent NIH3T3 in a well of the 24-well culture plate was recovered in 100 ul. 10 ul of which

were used in the PCR reaction, which is carried out in the following buffer : 10X standard PCR buffer including 25 mM of $MgCl_2$ (Perkin-Elmer/Roche MS); 100 nanograms (ng) of each primer; 2 μl of dNTPs 10 mM (equimolar mixture of each dNTP at an initial concentration of 10 mM, i.e. 2.5 mM of each); 2 units of cloned Taq polymerase (Perkin-Elmer/Roche MS) for 40 cycles, a single unit for 25 cycles; in a final volume of 50 μl.

Two pairs of primers were used.

The oligonucleotide sequences used are :

1°)   - for the first pair :

5' CTGCTGACGGGAGAAGAAAAAC-3'   5' CCCGCTCAGAAGAACTCGTC-3'

2°)   - for the second pair :

5' GACGAGTTCTTCTGAGCGGG-3'   5' GATCTGAACTTCTCTATTCTTG-3'

The size of the amplified sequences is in the case of the first pair , end-gag/two thirds proximal NeoR gene : 900 bp; and for the second pair, one third distal NeoR gene/proximal half of 3' LTR : 610 bp.

Denaturation 5 min at 94°C; 40 cycles on GeneAmpPCR 9600 with denaturation 30" at 94°C; annealing 15" at 55°C and elongation 30" at 72°C; followed by a terminal elongation step of 10 min.

The samples (15 μl out of 50 μl) were deposited on a 1.2% agarose gel (Seakem, FMC) and were subjected to horizontal electrophoresis for 45 min at 80 volts; the detection of the signal based on the analysis of the intensity of ethidium bromide (BET) fluorescence.

6 - <u>Determination of the infective titers</u>

Each of the clones tested for its capacity to infect NIH3T3 was amplified and optionally frozen prior to the analysis of the efficiency of infection by PCR.

After PCR two principal clones were selected and amplified in order to infect NIH3T3 according to a standard procedure

1 ml of 16 hours culture supernatant was taken at confluence from each producing clone on a dish 35 mm in diameter, filtered through a 0.45 μm filter in order to remove productive cells in

suspension. The supernatant was placed in contact with NIH3T3 cells at 50% confluence on culture dishes of the same diameter (35 mm) in the presence of polybrene at a concentration of 8 µg/ml of medium. The cells were incubated for about 2h30 at 37°C; the medium was shaken every half hour. Three volumes of fresh medium were added after 2h30.

INFECTIVE VIRAL TITERS

Successive dilutions of the primary supernatant were used to infect NIH3T3 cells; undiluted supernatant and supernatant at dilutions 1/10, 1/1000 and 1/100000. Two days after infection the cells were trypsinized, subcultured at about 1/20 on three culture dishes 100 mm in diameter and placed under selection by the addition of geneticin (1 mg/ml) to the supernatant.

This experimental procedure was made more stringent in the sense that : on the one hand, the drug was added very quickly after the infection; and, on the other, the direct placing in selection without trysinization of the cells prevents the artificial multiplication of the number of resulting colonies, the daughter cells derived from an infected cell remained grouped together and formed only one colony in situ. Conversely, when the cells are trypsinized, the daughter cells spread on the recipient culture dish and form artificially independent colonies which, if they are counted, artificially multiply the titer.

SOUTHERN BLOT

Two days after infection by the undiluted supernatant the NIH3T3 were trypsinized, subcultured at about 1/20 on three culture dishes 100 mm in diameter, one of which is subjected to selection by geneticin.

At confluence, the genomic DNA of the infected cells for each of the two clones after or without selection is extracted then quantified.

The DNA was digested by two restriction enzymes, PstI and KpnI, in order to carry out a Southern blot. After control of the

of DNA in each well, the transfer was carried out on a nylon Hybond N membrane (Amersham). Hybridization was performed with a probe which included all of the viral LTR sequences flanked by 100 bases upstream and 100 bases downstream. The probe was labelled by primer extension (Feinberg and Vogelstein, 1983, 1984) with alpha-^{32}P labelled dCTP of specific activity of 5×10^8 cpm/µg.

The hybridization was carried out in a medium consisting of : 50% deionized formamide; $5 \times$ SSEP; $1 \times$ Denhardt's; 5% dextran sulfate and 0.2 mg/ml of sonicated salmon DNA for 20 hours at 42°C. Brief rinsings were carried out in a solution of low stringency : $2 \times$ SSEP/0.1 %SDS, 5 min at room temperature and 10 min at 65°C; followed by exposure for 3 days to Kodak-XAR-5 films at -80°C with LI-Plus intensifying screens (Dupont-NEN).

7 - Search for the production of helper virus

This search was carried out by a mobilization test on 3T3BAG lines (Danos et al., 1988; Danos, 1991).

The 3T3BAG cells were initially infected by the undiluted supernatant of infected packaging lines. Several successive cycles of infection of unexposed 3T3BAG with the supernatant of previously infected 3T3BAG were carried out to sensitize the test.

RESULTS

1 - Construction of the retroviral vector FOCH29

The viral strain FB29 of the Friend murine leukemia virus was isolated (Mathieu-Mahul et al., 1982) and the genomic DNA of the integrated provirus was cloned in pBR322 (Sitbon et al., 1986). This genomic DNA has been completely sequenced (Perryman et al., 1991). The genomic fragment ClaI-PvuII of 2120 bp was cloned in pBR322. This fragment contains the last nucleotides of the sequence coding for the p15E of the viral envelope, all of the Long Terminal Repeat (or LTR) and 3/5 of the gag sequence. It constitutes the matrix of the architecture of the vector.

After replacement of the ClaI site by a EcoRI site the EcoRI-PvuII fragment was subcloned in the EcoRI-SmaI of the polylinker of pUC19. This clone was, on the one hand, kept intact to form the basic architecture of the vector including : the upstream LTR or 5' LTR, the binding site for the initiator of viral transcription (primer binding site or PBS), the packaging sequence, the gag sequences and the segment of the polylinker of pUC19 treated by EcoRI/SmaI digestion; destined for the insertion of the genes of interest.

On the other hand, a BamHI-BamHI fragment was derived by replacing upstream the EcoRI site by a BamHI site; and by taking advantage downstream of the endogenous BamHI site of the virus, situated immediately downstream from the donor splice site. This fragment was introduced into the basic framework of which the HindIII site of the polylinker has been replaced beforehand by a BglII site generating ends cohesive with those generated by the BamHI enzyme.

The marker gene derived from the retrotransposon Tn5 which confers resistance to neomycin (NeoR) was introduced between the two LTRs. After transformation on a strain of supercompetent bacteria of dominant negative recombinase phenotype in order to prevent possible reorganization of the sequences present, the transformants of the expected configuration were selected on the basis of the extended restriction map exploring all of the construction. One of them, designated pFOCH29, was then amplified and purified in order to have available an adequate source of material destined for the transfection of helper lines producing viral particles.

2 - Isolation of producer clones of defective virus

Transfection of psi-CRIP packaging lines : the plasmid pFOCH29-Neo was introduced in to the amphotropic packaging line psi-CRIP described by Danos et al. (1988) by transfection using calcium phosphate precipitation according to the standard procedure without carrier DNA.

After subjection to selection by geneticin, 40 of the colonies formed were taken and the culture supernatant was used to infect mouse fibroblasts (NIH3T3). The primary selection process of a

series of the most highly productive clones of packaged defective viral particles was based on the use of the gene amplification procedure by means of the polymerase chain reaction. The viral integration is analysed by PCR on a lysate of NIH3T3 which had reached confluence.

Two distinct PCR primer couples were used : 1° a first couple amplifying the terminal segment of the gag sequences included in the construction and the proximal two thirds of the gene for neomycin resistance ; 2° a second primer couple amplifying the distal third of the gene for neomycin resistance and the half of the downstream LTR (3').

Four clones were selected on the basis of a more intense PCR signal than the other 36; repetition of the procedure confirmed the initial data indicating that for two clones the signal was emitted markedly more intensely. These two clones were amplified and the culture supernatant of the producer cells was then used to infect NIH3T3 on a larger scale for the purpose of evaluating the efficiency of the construction in quantitative terms.

3 - Evaluation of the producer clones

Quantitative PCR

A semi-quantitative analysis by PCR was performed by using the primer couple amplifying the region corresponding to the distal third of the NeoR gene up to the median part of the downstream LTR (3' LTR). For each clone 1 µg and 3 µg of genomic DNA extracted from NIH3T3 cells infected by an undiluted supernatant after or without selection by neomycin were used. Each assay was performed in duplicate. Several dilutions of the plasmid pFOCH29-Neo were tested in parallel calculated such that they correspond to 0.1, 0.5 and 1 copy of transgene for the equivalent of 1 µg of genomic DNA, i.e. 0.115 pg, 0.575 pg and 1.15 pg respectively for a plasmid of 7164 bp.

The PCR was carried out for 24 cycles, which still corresponds to an exponential phase of the reaction. The reading was performed by computerized densitometric analysis (Cybertech) of ethidium bromide fluorescence (cf. Table).

In the case of the first clone a significant difference was observed between the intensity of the signal obtained from selected and non-selected infected cells; more clear-cut on the samples of 1 µg (70% of the signal with respect to the selected) than on those of 3 µg for which the detection system was saturated by the intensity of the signal.

In the case of the second clone, there is no difference in intensity of the signal between selected and non-selected cells, neither for the 1 µg samples nor the 3 µg samples. This suggests that a percentage of the NIH3T3 cells close to 100% had been infected by the undiluted supernatant of this producing clone. The high degree of infectivity of this clone was moreover suspected by the observation of an absence of cell mortality when the NIH3T3 infected with the culture supernatant were subjected to selection by neomycin.

Southern Blot

The DNA of the infected NIH3T3 cells was subjected to hydrolysis by two restriction enzymes : KpnI and PstI. The expected size of the bands after viral integration varies depending on the probes used. In the case of the enzyme KpnI which cuts within the LTRs and in the middle of the polylinker of pUC19, a LTR probe reveals a constant fragment size of 3610 bp and two fragments of variable size depending on the proximity of the endogenous genomic KpnI sites to the integration site; a probe derived by PCR with the primers distal third NeoR/proximal LTR segment, a fragment of the same size (3610 bp) is expected and only one of the two other fragments variable from one integration to another.

In the case of the enzyme PstI which cuts twice in the median part of gag and once in the polylinker of pUC19, after integration the fragments identified by the former two probes should be of variable size; a probe generated by PCR from the second primer couple identifies a constant fragment of 790 bases.

Several dilutions of the plasmid pFOCH29-Neo digested by KpnI and PstI were analysed on Southern blot; these dilutions correspond to 0.1, 0.5 and 1.0 copies of plasmid, respectively, for 10 µg of genomic DNA.

Furthermore, the DNA of the infected cells not selected by neomycin was systematically placed side by side in order to quantify the infectivity of the construction; the cells which had undergone selection constituted an infection control of 100%.

Titration : Infective viral titers by viral dilutions

Successive dilutions of the primary supernatant were used to infect NIH3T3; undiluted supernatant, supernatant diluted 1/10, 1/1000 and 1/100000. The cells are infected by a viral supernatant in the proportion of 0.5 ml per well 35 mm in diameter : the selection drug is added precisely 20 hours after infection directly on to the culture dish without trypsinization of the cells. The infective titer selected corresponds to the number of colonies observed for the last dilution at which colonies appear , multiplied by the inverse of this dilution.

In the case of the first clone the titer of inital producer cells is 2×10^6 pfu/ml. In the case of the second clone the titer is 10^6 pfu/ml.

The two producer clones were frozen normally in order to conserve initial passages on the one hand; and on the other, maintained in continuous culture for several months. The successive titrations (dilutions of the viral supernatant) made it possible to identify a progressive diminution of the titers. In the case of the first clone, the titer passed from more than 2×10^6 to only 10^1 in the interval of two months' continuous culture; this drastic fall in the titer was accompanied by a change in the growth of the cells in culture with a concentric appearance and ease of detachment. In the case of the second clone, the titer passed from more than 10^6 to 10^5 in an interval of two months' of continuous culture, to diminish to 10^3 -10^4 after three and a half months; this moderate fall in the titer was not accompanied by any change in the morphology or growth of the cells in culture.

4 - Helper activity assay on 3T3 BAG

This research was conducted by the mobilization test on 3T3 BAG lines (Danos et al., 1988; Danos, 1991).

The 3T3 BAG cells were infected initially by the undiluted supernatant of infected packaging lines. Several successive cycles of infection of unexposed 3T3 BAG with the supernatant of previously infected 3T3 BAG were carried out to sensitize the assay, which proved to be negative. Furthermore, colonies of cells resistant to neomycin could not be detected after placing the unexposed NIH3T3 in contact with the supernatant of infected NIH3T3 selected by neomycin.

5) INTEGRATION SITES

Human and non-human primate cells were used to identify the number of integration sites of the virus after infection. The mouse cells provide indications of very moderate quality in as much as there exists in these cells a significant background associated with multiple integrations of retroviral or retroviral-like sequences.

For this purpose, monkey VERO cells were infected with several dilutions of viral supernatant. In the case of the dilution 10^{-2}, independent clones were obtained; each one having been initiated from a single profile of viral integration. The use of a restriction enzyme which cuts within the viral construction, on the one hand, and in the genomic DNA of the cell host at variable distances from the integration sites, on the other, made it possible to obtain as many restriction fragments of variable size as there were integration events. In this case, the enzymes XbaI or SalI were used.

6) STABILITY OF THE VIRAL TITERS - MASTER BANK SYSTEM

A homogeneous stock of cells producing virus was constituted and extensively controlled for the absence of viruses competent to replicate by the following methods :
- Mobilization test on 3T3 BAG cells
- Amplification on NIH3T3
- Intraperitoneal inoculation of newborn mice,

in order to study a possible _in vivo_ pathogenesis.

Starting from this cell bank ("Master Cell Bank" MCB) a working cell bank was constituted. The viral titers obtained remained stable for several months and were of the order of 20 x 10^6 to 3 x 10^7 cfu/ml (the initial dilutions having been grown systematically only at 10^{-6}). Results were also obtained with an assay including a dilution at 10^{-7}. The titers are remarkably stable for several months at this level of intensity.

DISCUSSION

1° Construction of the virus

The viral construction was based on the principle of conservation of critical sequences such as the PBS, the packaging sequence, the donor splice site and also on the conservation of a long gag segment, the maintenance of which contributes to the stabilization of the transcripts as other authors have shown (reviewed by McLachlin et al., 1989).

Several other constructions derived from the same retroviral skeleton have been produced previously; including an initial one in which the 3' LTR was flanked upstream and downstream by longer retroviral sequences and in which two polylinkers of pUC19 and pUC18 were present. Great instability and modest infectivity resulted from this configuration.

A version similar to that of FOCH29 was constructed; similar in all points except for the insertion of a large sequence including the acceptor splicing site of the "clone 57" strain of the Friend virus (Sitbon et al.). The resulting plasmid was designated pFOCH29SA-Neo. The infectivity of this construction proved to be less marked than with pFOCH29-Neo. Nonetheless this difference was only perceived on the data of the primary screening by PCR.

2° Selection of the producer clones

The primary selection of the producer clones by the procedure described in the Results section exploits the polymerase chain

reaction; the conditions used limit the resolving power of the method to a threshold which corresponds approximately to a minimal retroviral titer of 10^4 pfu/ml. The potential disadvantage of this procedure is linked to the not absolutely quantitative character of the PCR, in particular for forty amplification cycles; consequently, there is a not inappreciable probability of missing highly productive clones. In order to compensate in part for this disadvantage a primary selection procedure based on two independent primer couples for PCR was adopted.

3° Efficiency of the infection and stability of the virus

The use of two primer couples for PCR each generating amplimers of expected size also makes it possible to verify the absence of gross rearrangements of the viral genome after integration.within the segments analysed

This element is best checked by the Southern blot which verifies the absence of major rearrangements in the absence of inadequate bands both in number and size ; as well as the absence of multiple integration events within the cells derived from the same clone which would suggest the presence of helper virus.

Finally, the absence of major rearrangements likely to adversely affect the construction is best appreciated by the functional assay; demonstrating the acquisition of a phenotype resistant to neomycin after chromosomal integration of the marker gene.

The efficacy of the infection has been assessed here by means of three essential parameters : 1°) the conventional viral titration which made it possible to demonstrate titers higher than or equal to 10^6 pfu/ml.

2°) the Southern blot comparing the intensity of the signal obtained after hybridization on a hydrolysate of 10 µg of genomic DNA derived from infected cells without selection and after selection by the drug to which the product of the transduced gene confers resistance. If theoretically calculated plasmid dilutions were added

In fact, the plasmid dilutions are such that a minor inaccuracy in handling may lead to the erroneous conclusion of a significant difference.

All of these elements added to an experiment (3°) of semi-quantitative PC R converge to indicate that the undiluted supernatant of the second clone infects the mouse fibroblasts with an efficacy close to 100%.

However, instability of the titers was observed with a rapid drastic loss in the case of the first clone and a much more gradual diminution for the best. This phenomenon is a commonplace observation in the handling of producer cells which seem to lose their initial packaging capacities with successive passages. The initial production of large quantities of cells and their careful freezing makes it possible to compensate for this disadvantage. Nonetheless the titer must be systematically and repeatedly checked.

Furthermore, an amphotropic producer line was selected. However, the initial transfection of the ecotropic producer cells would have made possible the infection of amphotropic lines, possibly repeated reciprocally and repeatedly according to the "ping-pong" procedure (McLachlin JR) (by means of the supernatant filtered to prevent a possible mixing of the two cell types); this latter might contribute not only to increasing the infectivity of the resulting producer cells but also to stabilizing the retroviral titers. However, it was observed that systematically helper viruses are produced and this method can not be used in animals or in the clinic.

The quite special efficacy of this construction deserves to the emphasized; all the more since the backbone of the FB29 strain of the Friend virus differs from that which was used to establish the packaging lines. The safety of handling is still further improved since the risks of potential recombinations producing replication-competent virus are further reduced.

The introduction of a marker gene or any sense or antisense cDNA sequence or genomic DNA fragment of limited complexity (limited number of introns) of a size less than or equal to 7 kb from which will be derived a cDNA whose introns will be expelled in the

targets may be achieved starting from the previously described procedure.

In the case in which the preservation of intron sequences seems essential to the production of stable transcripts, the genomic fragment of a size less than or equal to 7 kb will need to be introduced in a reverse transcriptional orientation with respect to viral transcription, at best by using a version of the construction in which the 3' LTR is deleted from the U3 and in which the genomic fragment is placed under the transcriptional dependence of a promoter and/or enhancer in the same orientation in order not to have competition between the sense transcripts dependent on the viral LTRs and antisense transcripts dependent on the added promoter.

The introduction of a deletion of all or part of the promoter or enhancer sequences of the U3 region of the 3' LTR offers guarantees of considerable safety after integration of the retrovirus. In this case, the expression of the transgene will need to be placed under the dependence of an exogenous promoter and/or enhancer within the construction.

4) - <u>Integration sites</u>

The study of the number of integration sites of the virus after infection of primate cells has provided very relevant information concerning the physiology of the defective viruses with high infective titers and concerning the safety of use of these viruses in therapeutic applications in man.

B)   <u>ADDITIONAL RETROVIRAL CONSTRUCTIONS</u>
B1)   <u>GENERAL CONCEPTION AND VERSATILE USE</u>

1)-   SELF-INACTIVATING RETROVIRAL VECTOR (SIN);
      designation : FOCH29-del U3 shown in Figure 6.

In the light of the physiology of the replication of the genomic genetic material of the retroviruses, the modifications intended to delete the enhancer viral sequences of transcription were introduced at the 3' LTR: viral replication led at the level of the

target cell of the infection to an integrated provirus whose two LTRs were generated from sequences of the initially 3' LTR.

This deletion was hence made on the plasmid containing the BamHI-BglII fragment including the 3' LTR sequences. The resulting BamHI-BglII fragment shortened by 339 bases was excised and ligation with the plasmid containing the 5' LTR and the adjacent sequences was carried out according to the same procedure as for the basic vector. In fact, the enhancer sequences of the 5' LTR were left untouched in order that the viral transcription could occur and form "readthrough" transcriptions; infectious virus particles were thus formed at the level of the packaging line.

The construction design chosen leaves in place the TATA box but removes the CAAT box (Yu et al., 1986) by cutting using the enzyme BssHII (unique site at 8203 of the sequence of the native virus) which excises the CAAT box; the protruding 5' cohesive ends generated by the enzyme BssHII are made "blunt" by filling in using the Klenow fragment of the DNA polymerase I in the presence of deoxynucleotides.

The restriction enzyme used upstream was :

- either EspI (= iso-CelII) at position 7864, situated immediately after the start of the inverted repeats (IR). The protruding 5' cohesive ends generated by the enzyme EspI were made "blunt" by filling in using the Klenow fragment of the DNA polymerase I in the presence of deoxynucleotides.

- or EcoRV at position 7984 in the middle of the direct repeats which the cut interrupts; the ends generated by the enzyme EcoRV are blunt.

Since the ends upstream and downstream have both been made blunt, direct ligation was possible to close up the construction, henceforth bearing a large deletion in the U3 region.

The first version delU3 (deletion EspI at 5'/Iso CelII at 3') was studied in functional terms : viral infection and integration and residual expression. The deletion created here has removed practically all of the U3 sequences of the virus. This is ideal in terms of safety of the retroviral construction.

We have been able to show that viral transcription was not adversely affected in the packaging lines by introducing the beta-

construction. In fact, the producer cells took on a blue colour after transfection which confirms not only the presence of the gene borne by the retroviral construction but also the expression of this transgene.

The viral integration was investigated by molecular methods, in particular by PCR using the following primers : oligo-SENSE situated in the beta-galactosidase gene with the following sequence : 5' - CGA CTC CTG GAG CCC GTC AGT ATC -3'; oligo-ANTISENSE situated in the viral LTR, overlapping between R and the start of U5 (LTR-508) : 5' - CAG CGA GAC CAC GAG TCG GAT GC - 3' in a region which has been prepared by EspI/BssHII deletion.

Alternative constructions consist for example of inactivating the viral enhancers as in the case of the deletion with EcoRV, even of making shorter deletions leading to the retention by the virus of a background transcriptional activity in the target cells of the infection. On the other hand, a deletion removing the TATA box in addition to the sequences described in the version called the first version of 1U3 represents an additional security device ; however, considerable reductions of titers may occur when such constructions are formed (Yee et al., 1987).

SIN CONSTRUCTIONS WITH INTERNAL PROMOTERS

An internal promoter which can be used to create these constructions is for example the promoter of the receptor for the EGF (Epidermal Growth Factor) derived from the plasmid pERCAT2DE(c) (Maekawa et al. 1989) : only the promoter sequences situated upstream between the nucleotides -2200 and -15 (EcoRI-SstI fragment of 2.2 kb) were selected. The enhancer sequences downstream contained in the plasmid pERCAT2DE(C) were not included. Another promoter is the ubiquitous PGK promoter (phosphoglycerate kinase).

2) - DEFINITIONS OF THE EXISTING CONSTRUCTIONS

2° - 1 A construction was made with a still more reduced LTR upstream from the 3' LTR in particular; designation FOCH29-PL (for FOCH29 pure LTRs) shown in Figure 7

This construction made it possible to assess the advantage in terms of genetic stability of the excision of the 140 bases, including 104 of the end of the envelope upstream from the viral LTRs.

The construction was made from the plasmid pUC19 including the EcoRI-PvuII fragment described in part B1-1 : enzymatic cutting by the restriction enzyme EspI (or IsoCelII) at position 7864 (namely +23 of the viral LTR). At the 5' end the bases generated by a EcoRI cut were artificially added to a double stranded synthetic oligonucleotide complementary to the 23 bases of the LTR (140 bases, 103 of which are bases of the envelope). At the 3' end the oligonucleotide is complementary to the cohesive SpeI ends. The oligonucleotide sequences are the following : oligo-SENSE 5'- AAT TCA ATG AAA GAC CCC AAA TTG C -3'; oligo-ANTISENSE 5' - TAA GCA ATT CGG TGG GGT CTT TCA TTG -3'.

2° - 2 GAG shortened while preserving intact the packaging sequences; designation FOCH29-SG (for FOCH29 short GAG) shown in Figure 8.

The basic construction FOCH29 leaves half of the GAG sequences in place; the transcription and probably the translation of MAp15 and pp12 being preserved.

The preservation of a significant portion of GAG has been described as being beneficial for the stability of the retroviral construction. This may however represent a disadvantage in terms of the space available for the exogenous sequences to be vectorized, on the one hand; and on the other, in terms of safety of the construction, the probability of promoting recombinational events with retroviral sequences to generate replication-competent viral particles being significantly increased.

FOCH29 contains scarcely more than half of the GAG sequences of the FB29 strain and offers the advantage of very remarkable efficacy.

A culture of the producer clones of FOCH29, maintained for 18 months, did not lead to the production of an amount of recombinations with retroviral sequences generating replication-competent viral particles and thus detrimental to the safety of its use

In this perspective of improving viral safety, an alternative construction was prepared in which the GAG of FB29 is cut at the unique AhaIII (or isoDraI) site at position 1031; only a quarter of the GAG sequences are then conserved. The cut generated by AhaIII having blunt ends as for PvuII used for the cloning of FOCH29, the construction was made exactly superposable, the cloning upstream not being modified; downstream, the blunted ended SmaI site of the polylinker of pUC19 was used.

2° -3 Constructions including IRES (for Intra Ribosome Entry Site or Ribosome Landing Pads) leading to polycistronic messenger RNAs.

A retroviral construction including IRES is in principle designed for the transfer of several genes of interest for which it is desired that the level of transcription is balanced since it is initiated from a single promoter (Morgan et al., 1992); the most illustrative example is that of the transfer of the sequences coding for the different chains of a functional molecule in a heterodimeric or trimeric context.

A polycistronic vector was constructed for the transfer of the two chains of interleukin 12 or IL12, p35 on the one hand and p40 on the other.

The p35 fragment introduced was the following fragment : at 5' : PstI at position +187 of the sequence; of the two ATG (Met) codons in phase (positions 100-102 and 202-204) , that used was at position 202-204; and at 3' : EcoRI at position +1065 (stop codon TGA at position 904-906).

The p40 fragment introduced lacked the flanking 5' or 3' sequences : XbaI site at position +1 of the sequence (ATG at position +9); and at 3' : EcoRI at position +1007 (stop codon TAG at position 993-995). The complementary DNA to p40 has an optimized AUG codon (Sequence Kozak CCATGG ; corresponding to the NcoI restriction site).

Two types of IRES were used (Borman et al., 1992; 1993);
- IRES derived from poliovirus which required accurate positioning; ribosome binding site at 560, AUG binds to position

This is the fragment KpnI (position +70) / BalI (position +630) which was used : either upstream of p35 (FOCH29-NIRIL12); or upstream of the gene for resistance to neomycin (FOCH29-IL12-30IR4ON).

- IRES derived from EMCV (for EncephaloMyoCarditis Virus), in which the IRES sequences derived from EMCV require extremely accurate positioning, in phase, of the gene to be expressed (IRES sequences derived from EMCV are sold under the name of pCITE for CAP INDEPENDENT TRANSLATION ENHANCER (Novogen, USA), very similar to those which were used here). In the light of the optimized character of the AUG of p40, it is this chain which was associated with the IRES-EMCV whatever the construction; the EMCV fragment used comprises a EcoRI site upsteam (position +280) and a NcoI site downstream situated just after the AUG (leave a "C" residue which corresponds to the second base of the fourth codon).

Three constructions were made :

Figure 9   1) FOCH-IL12 : p35 / IRES of poliovirus
                     / p40 : LTR promoter

Figure 10   2) FOCH29-NIRIL12 : neomycin resistance gene / IRES
                  of poliovirus / p35 / IRES of EMCV / p40

Figure 11   3) FOCH29-30IR40N : p30 / IRES of EMCV / p40 / IRES
                  of poliovirus / neomycin resistance gene

These constructions all have resort to an intermediate construction in pUC18 in which :

1°) a NotI site was introduced at each of the ends of the polylinker, in HindIII upstream and in EcoRI downstream;

2°) a SalI site was introduced downstream from the polylinker between the EcoRI site and the NotI site

3°) a base motif ("pUC18-base") was cloned comprising : p35 / IRES of poliovirus / p40. Starting from this base motif, the construction FOCH-IL12 was obtained by excision by means of NotI digestion; ligation is carried out between the fragment obtained and the vector FOCH29 opened by NotI for which a linker was introduced at the XbaI site.

The constructions FOCH29-NIRIL12 and FOCH29-IL12-30IR40N were obtained after addition respectively : of the block neomycin gene resistance / IRES of poliovirus at the SphI site upstream from the "pUC18-base"; and the block IRES of poliovirus / neomycin resistance gene at the SalI site downstream from the "pUC18-base".

2° -4 Construction GAG-POL to contribute to the development of an original packaging line.

The nucleocapsid proteins and the reverse transcriptase are derived from sequences of the FB29 strain in the complementation line.

The packaging lines presently available and derived from mouse cells possess the following disadvantages :

- Co-packaging, concomitant with defective constructions, of endogenous retroviral sequences (MCF, VL30, even retrotransposons). These co-packaged sequences are thus also likely to be integrated after infection of the target cells of the transfer.

- Expression of complementation proteins, in particular the envelope, piloted in these lines by a retroviral LTR. Although the lines of the third generation use complementation retroviral constructions comprising several mutation or deletion sites, the preservation of LTR sequences is in itself a potential disadvantage; in fact, they are capable of giving rise to genetic recombination with the defective constructions to be complemented.

All of these elements have led to attempts to improve the safety of conditions of genetic transfer with retroviral vectors. The packaging line was developed on the principle of the third generation lines; i.e. with fragmentation of the sequences coding for the complementation proteins in two parts (two successive transfection steps).

2° -4-1 In the first stage, the steps implying the use of sequences derived from the FB29 strain of the Friend virus are described : in particular, development of the basic cell "DOGP29".

DOGP29 was obtained by transfection of the construction LTR-SD-deletion psi-GAG / POL detailed below (and co-transfection with selection gene, resistance to phleomycin) on dog fetal cell optimized according to the following criteria : 1 - absence

of endogenous retroviruses; 2 - adherent cell; 3 - rapid growth; 4-stable and homogeneous morphology; 5 - easily transfectable; 6 - very high number of passages tolerated (intensive artificial passages for assay); 7 - optionally capable of sustaining LTC-IC (Hemato).

A Master Cell Bank System is created from the clone of dog cells selected according to the intensity of synthesis of viral complementation proteins and the stability of expression of the reverse transcriptase (POL).

The complementing gag/pol construction for the nucleocapsid proteins and the reverse transcriptase is derived from the FB29 strain of the Friend virus.

The basic construction was assembled from the construction described in the paragraph in which the LTR was left in place or replaced by the sequences of the RAR-beta promoter.

A large deletion of the packaging sequences situated upstream from the sequences coding for the gag capsid nucleoproteins (starting at + 619) is carried out as follows : SpeI (or IsoceII) cut, unique site at + 280; and PstI at + 560-561 which removes 280 bases. A synthetic linker SpeI-PstI is synthesized: 5' -CTAGTGCA-3' and annealed to the plasmid, cut again by HindIII. Ligation is then carried out with the third fragment PstI-HindIII including the major part of the GAG and POL sequences.

Then, in a second step (after transformation and selection of the positive recombinants), the PstI-PstI (561-737) including the ATG of GAG was cloned at the PstI site in its original position in order to reestablish the totality of the GAG sequences; the orientation of the cloning of this small symmetric PstI-PstI fragment is established by enzymatic digestion with HaeII (position 720; a second site is situated at 4291 but does not disturb the orientation) / AhaIII (unique site in the entire FB29 sequence at position 1031) or EspI (unique site in th entire FB29 sequence at position 7864 of the LTR). As a result of the method used for the cloning at the HindIII site of pUC19 of the DNA sequences corresponding to the entire FB29 genome, the end of the POL sequences was recovered as follows : initial cut by HindIII (5060) and SnaI (= iso Bst1107I) (unique site at 6335); after purification, this fragment was cut again by SmaI (6079) to produce a fragment of 1019 bases comprising a minimum of envelope sequences (244 bases). This fragment was

subcloned in the HindIII-HincII of the polylinker of pUC18. The polyadenylation signal of SV40 was juxtaposed downstream (excised from the plasmid pCRIPgag-2, Danos and Mulligan, 1988) from the construction. The POL and polyA sequences were excised as a unit from pUC18 and ligation was carried out with the plasmid pUC19/partially deleted LTR /del-psi/GAG / 2/3-POL, described in the above paragraph.

2°-4-2 The conventional amphotropic envelope sequences , similar to those used in the psi-CRIP line were used for sequence complementation with transcription under viral 5' LTR with a polyadenylation site downstream from SV40 as in the psi-CRIP line (Danos and Mulligan, 1988).

B.2. <u>INCLUDING GENES OF INTEREST</u>

Whether they are constructions :

- using the basic vector with its native LTRs or the SIN version from which the viral enhancers are deleted

- using various internal promoters for the constructions derived from the SIN vector

- positioning the gene of interest in a sense or antisense transcriptional orientation with respect to viral transcription.

1° - cDNA coding for the beta-galactosidase with a nuclear localization signal (Figures 12, 13)

2° - cDNA coding for gp170 derived from the gene for pleiotropic resistance to cytotoxic drugs (ex insert derived from pMDR1) (Figure 15)

3° - Genomic sequences and cDNA coding for metallothionein IIA (Figures 16, 17, 18, 19)

4° - cDNA of the FACC gene with or without its promoter elements, deficient in patients suffering from Fanconi disease of the complementation group C

The FOCH29 retroviral backbone was used :

a)- either in its native version with transcription defined by the activity of the viral Long Terminal Repeats (LTRs). This construction makes it possible to assess the efficacy of the LTRs of the Friend virus to express the FACC gene in hematopoietic cells.

b)- or in the del-U3 version of the viral enhancer which makes it possible to test the advantage of a transcriptional command by an internal promoter derived from a ubiquitously functioning gene with a moderate but stable basic function : it is either the promoter of the FACC gene itself or the MT IIA (metallothionein IIA) promoter, or the PGK (phosphoglycerate kinase) promoter. The construction using the promoter of the FACC gene is preferred provided the level of expression obtained is compatible with a phenotypic correction.

Choice of the cDNA :

Three different complementary DNAs correponding to three types of messenger RNAs were cloned for the complementation group C of Fanconi's disease; the open reading frame is identical irrespective of the complementary DNA considered . (Strathdee et al, 1992).

One of the messengers is largely predominant in the cells in culture. Its 5' end comprises only a part of the exon -1; on the other hand, the non-coding 3' end is very extensive. The flanking 3' sequences seem to have a decisive importance for the stability of the transcripts.

Whatever the complementary DNA chosen, the latter is excised by BamHI/XhoI cutting and introduced into the retroviral constructions in an <u>antisense orientation</u> as follows : upstream, XhuI is cohesive with the ends generated by SalI cutting ; downstream the SphI site is made blunt, thus adapted to the BamHI site itself also made blunt.

1- Most of the complementary DNA is 4.5 kb (size corresponding approximately to the upper limit for a retroviral vector).

2- The DNA complementary to one of the other two messengers was also used in the <u>sense orientation</u> within the enhancer-free retroviral construction (del-U3) to test the potential advantage of using flanking 5' sequences and the -1 exon for the expression of the FACC gene; the cloning is then achieved by XbaI cut made blunt upstream to adapt to BamHI -blunt, the SalI-XhoI adaptation being made downstream.

3- Finally, more simply, the complementary DNA corresponding to the regions coding for the FACC gene lacking flanking regions at 5' and 3' was introduced :

- either in the native retroviral vector in the sense orientation with transcription governed by the retroviral LTRS

- or in the enhancer-free retroviral vector in the sense orientation with transcription governed by a ubiquitous metallothionein or PGK (phosphoglycerate kinase) promoter.

5°- cDNA of the PLP gene deficient in patients suffering from the Pelizaeus-Metzbacher disease (Dautigny et al., 1986; Hutson LD et al., 1989; Morello et al., 1986).

A construction (Figure 24 was assembled for the time being in order both to express the PLP gene under its highly specific tropic natural promoter and inducible by glucocorticoids and to be able to monitor oligodendrocytes or Schwann cells expressing PLP in vivo after stereotaxic implantation in the brain.

For this purpose the gene coding for beta-galactosidase equipped with a nuclear localization signal (nls-Lacz) was placed under the direct dependence of the LTR. The PLP promoter / complementary DNA - PLP sequences are placed downstream in the polylinker. The BamHI-BamHI fragment of nls-Lacz was cloned in the BamHI site of the polylinker of pSPT18. The AluI/BamHI and BamHI/EcoRI fragments (treated with Klenow in the presence of dNTPs) including respectively the PLP promoter and the PLP complementary DNA were cloned after ligation to the SmaI site upstream (in the case of AluI) and EcoRI site downstream in pSPT18.

The entirety of the insert is excised by EcoRI downstream, the end of the insert is treated with Klenow (blunt end) then SalI upstream; and adapted to the polylinker of FOCH29 upstream at SalI and downstream at SphI treated with Klenow (blunt end).

Other constructions in the del-U3 version of FOCH29 are planned including in reverse orientation the PLP promoter followed by a sequence comprising the first intron of the gene associated with the complementary DNA. The presence of this intron ought to improve the expression and stability of the transcripts and allow a possible alternative splicing PLP-DM20.

6°- cDNA of each of the chains of interleukin 12 : p35 and p40 in a polycistronic vector, construction described above as example of construction comprising IRES.

Other constructions may be obtained by including for example the following genes :

7°- TIMP : Tissue Inhibitor of Metalloproteinases
8°- TNF : Tumor Necrosis Factor
9°- IFN-gamma : gamma Interferon
10°- IFN-B : beta Interferon
11°- cytokine genes such as for example interleukin

C-   CELL TARGETS

The vectors of the invention were used to transfect different stem cells. As examples the following cells are cited :

a- HEMATOPOIETIC STEM CELLS OF HUMAN ORIGIN

The retroviral vector FOCH29 was initially constructed for the purpose of investigating a viral strain capable of infecting hematopoietic stem cells more effectively and of leading to a higher significant expression of the genes of interest in these cells than with the vectors currently used. This supposed effect is expected as a result of a particular tropism of the viral regulatory sequences, in particular U3 sequences of the LTRs.

The gene for neomycin resistance (neomycin phosphotransferase derived from from the transposon Tn5) was introduced to form the vector FOCH29-Neo previously described and used as gene marker.

For one year the optimal conditions have been studied for the transduction of CD84+ hematopoietic progenitors of human origin, selected according to various methods obtained either from 1l) blood of the umbilical cord, 2l) bone marrow (allogenic graft), 3l) stem cells of the peripheral blood mobilized by a combination of chemotherapy and growth factors.

1- Comparison of different procedures of viral infection

    1-1 Use of viral supernatant versus coculture (on packaging cells producing the virus continuously)

    1-2 Stimulation of the cells by different combinations of growth factors

2- Evaluation of the success of transfer :

    2-1 absolutely, whatever the stage of differentiation of the transduced cells, by means of biological and molecular methods

    2-2 in cells capable of representing pluripotential cells with the development of long-term cultures, in particular of xenogeneic stroma of mouse origin and sequential analysis of the clonogenicity of the cells.

The results (in detail below) obtained, in particular starting from viral supernatants, confirm the initial hypothesis of a remarkable efficiency of the retroviral vector on the hematopoietic precursors.

The details of the experimental protocols as well as the results obtained are described :

EXPERIMENTAL PROTOCOLS

1- COMPARISON OF DIFFERENT PROCEDURES OF VIRAL INFECTION

1-1. Use of viral supernatant versus coculture on packaging cells producing the virus continuously.

In fact, the use of cocultures presents major disadvantages for human uses relating to :

1) the potential contamination of the hematopoietic cells by xenogeneic cells and

2) the potential persistence of a multiplication of these producer cells in spite of their being irradiated (or their treatment by cytotoxic agents) prior to the use in coculture.

3) the significant increase of the risk of the generation of replication-competent virus as a result of the presence of sequences complementing the defective viruses, capable of inducing

endogenous retroviral sequences or accidental, contaminating viruses of the culture media (Temin et al., 1990).

A comparison in parallel was made of the infection of the same unique cell source (CD34+ progenitors selected either from a unique sac of blood of the umbilical cord; or from a cytapheresis) separated into two equivalent samples from all points of view; one of them was placed in contact with an adherent sublayer of virus producer cells grown to 80% confluence on 1% gelatin in the presence of polybrene at a concentration of 2 mg/ml for 48 hours; the other sample was placed in a culture flask and covered with viral supernatant (undiluted with additional culture medium) freshly harvested from cultures of producer cells at confluence and filtered through a 0.45 μm membrane (removing all of the cells likely to have been taken with the supernatant), in the presence of polybrene at the same concentration. The infection protocol by viral supernatant was based on a repetition of four cycles over 36 hours, i.e. two cycles per day at an interval of eight hours on two successive days. The supernatant was simply added to the culture well without the hematopoietic cells being handled or centrifuged.

The culture medium used for the viral infection irrespective of the mode of infection corresponded to the medium optimized for hematopoietic stem cells, namely Iscove's modified DMEM (GIBCO-BRL) supplemented with 10% fetal calf serum (Boehringer-Mannheim) and 10% horse serum (GIBCO-BRL).

Each parameter was simultaneously tested in at least two wells in duplicate.

After infection, the cells were planted for long-term culture on xenogeneic stroma.

1-2. Stimulation of the cells by different combinations of growth factors.

Minimized quantities of growth factors were used in order to preserve maximally the pluripotentiality of the hematopoietic precursors which might be infected, the objective being to best treat the cells already spontaneously in cycle.

A comparison has been made on the same cell source and under the same condition of infection of :

1°- DIFFERENT COMBINATIONS OF GROWTH FACTORS

(Stem Cell Factor : SCF; Leukemia Inhibiting Factor : LIF; Interleukin 3 : IL3; Erythropoietin : Epo; Granulocyte-Macrophage stimulting factor : GM-CSF), namely :
- SCF + LIF
- SCF + LIF + IL3
- SCF + LIF + IL3 + Epo
- SCF + LIF + IL3 + GM-CSF

2° - DIFFERENT CONCENTRATIONS OF THESE DIFFERENT FACTORS
- SCF : 50 ng/ml; 25 ng/ml; 10 ng/ml ; 5 ng/ml
- LIF : 10 U/ml; 5 U/ml; 1 U/ml
- IL3 : 10 U/ml ; 1 U/ml ; 0.1 U/ml
-GM-CSF 10 ng/ml

2- EVALUATION OF THE SUCCESS OF TRANSFER

2-1 All cells taken together immediately after infection, giving rise to an initial percentage of transfected cells on the basis of molecular and biological methods :

- CFU-GEMM (assay for the presence of mixed colonies Granulous-Erythroid-Monocytes-Megakaryocytes) according to two forms :

- without selection

- with pharmacological selection by means of neomycin used at initially high doses, namely 1 mg/ml.

- PCR (polymerase chain reaction) on individual colonies starting from the CFU-GEMM assays on methylcellulose subcultured on non-selected cultures; after subculturing the individual colonies were amplified to about 10^5 cells per well by stimulation with a cocktail of growth factors containing : SCF 50 ng/ml; GM-CSF 50 ng/ml; IL3 10 U/ml; and Epo 2U/ml. The cells were then lysed and analysed (cf protocol and PCR primers indicated in the part) .

2-2 in cells likely to be pluripotential cells

An assay of long-term cultures on xenogeneic stroma of murine origin (line MS5) was used and sequential analysis of the clonogenicity of the cells.

Long-term cultures on xenogeneic stroma, the adherent monolayer of which was produced beforehand on a film of 1% gelatin. These cultures were grown in "slide-flasks" of 9 cm^2 surface area (Nunc) for 60 days; each week half of the surface supernatant was removed and replaced by fresh medium containing a minimal cocktail of growth factors (Stem CEll Factor : SCF 5 ng/ml; Leukemia Inhibiting Factor : LIF 5U/ml; Interleukin 3 : IL3 0.1 U/ml and erythropoietin : Epo 0.1 U/ml).

The long-term cultures were grown in normal medium or in a selective medium with a stroma genetically manipulated by transfection with a PGK-neo plasmid in order to be made resistant to neomycin.

The evaluation of the long-term cultures was based on :

- the observation of the presence of islets of hematopoietic cells at the surface on the stromal cells (Cobblestone)

- the starting at defined times of sequential CFU-GEMM assays with or without pharmacological selection for the presence of the gene for neomycin resistance. The cells sampled were numbered and seeded in aliquots of 1000 cell per analysis well. The cells were derived either from the surface supernatant generally taken each week or from a supernatant withdrawn so as to detach also the hematopoietic islets adhering to the stroma (in particular on D60 when the long-term cultures were stopped) as follows : all of the supernatant was taken, the culture dish was then covered with 1x PBS without calcium for one to two minutes; this detached the partially adherent cells. This second supernatant was itself removed, pooled with the first and the whole was centrifuged at very low speed (1000 revolutions/minute) on a cushion of fetal calf serum which protected the most fragile cells. One part of the cellular pellet was planted for CFU-GEMM, the other cells being simply reseeded on an xenogeneic stroma for long-term culture.

- the molecular characterization of the viral integration was analyzed by sequential PCR assays on individual colonies taken from the CFU-GEMM (see above).

- when a sufficient number of cells could be obtained at the outset and when the long-term cultures remained very rich, one

RESULTS

1 - LONG-TERM CULTURES

The cultures were maintained successfully for 60 days without changing the stroma during this interval. At that date, the cultures were stopped and all of the remaining cells were seeded in CFU-GEMM. Colonies in CFU-GEMM assay seeded at 60 days from cells selected on neomycin were obtained. This confirms the efficient transduction of hematopoietic precursors capable of being maintained in long-term culture and the conservation of a genic expression for several months of the gene carried by the construction FOCH29 in the hematopoietic precursors.

2- COMPARISON OF DIFFERENT PROCEDURES OF VIRAL INFECTION

2-1 Use of viral supernatant versus coculture

The remarkable efficiency of four repetitive cycles of infection by supernatant during two days, without manipulation of the cells has been observed in comparison with coculture on a packing line for 48 hours.

These data were authenticated by :

1° The maintenance of long-term cultures in the presence of a selection by neomycin on stroma resistant to neomycin; at D60 the cell cultures infected by supernatant still remained productive whereas the wells infected by coculture were unproductive.

2° The CFU-GEMM assays with a number of colonies, in particular mixed colonies, 4 to 5 times more numerous after infection by supernatant.

3° The molecular evaluation of viral integration by PCR on individual colonies subcultured and amplified from the CFU-GEMMs. In the case of the initial assays a transduction of up to 90% was detected after infection by supernatant.

2-2 Stimulation of the cells by different combinations of growth factors.

Of the multiple different combinations cited in the experimental protocol section, the minimal cocktail of growth

factors (GFs) selected at minimal concentrations was the following : SCF 10 ng/ml + LIF 10 U/ml.

In fact, the significant long-term cultures after infection in the presence of IL3 at concentrations varying between 1U/ml and 10U/ml could not be maintained owing to a significant initial expansion without maintenance of a productive long-term culture.

Furthermore, no effect of the pretreatment by xenogeneic stroma except that a potential contamination by mouse cells was not observed.

These results thus show :

1° the feasibility of a productive retroviral infection without endangering the initiation potential of long-term cultures, starting from a viral supernatant but not from a coculture.

2° the efficiency of both the viral transduction of the hematopoietic precursors of human origin derived from the CD34+ cells by means of the construction FOCH29; and the expression of the gene of interest throughout the long-term culture starting from this construction in these same cells.

These elements attest to the feasibility of therapeutic uses by means of this retroviral vector in human hematopoietic stem cells; this ought to be confirmed by the results of a protocol of clinical experimentation of graft labelling.

  b-   EPITHELIAL CELLS
    1°- Cells of the vesicular epithelium
      - of rat, dog, monkey
      - of human origin
    2°- Cells of the mammary epithelium
      - of rat
      - of human origin
  c-   TUMOR CELLS
    1°-   Cells of vesicular tumor
      - of rat
      - of human origin
    2°-   Cells of mammary tumor
      - of human origin
  d-   ACCESSORY CELLS OF THE NERVOUS SYSTEM
    1°-   Oligodendrocyte precursors
    2°-   Schwann cells

These two types of cells of mouse origin were infected with the construction FOCH29-Neo in order to evaluate the neurotropic tropism of the retroviral LTRs; the expression of the gene of interest being governed by the retroviral LTRs.

The oligodendrocytes were derived from primary cultures; in the case of the Schwann cells, the line MSC80 was used. After infection, the MSC80 cells were maintained in continuous culture and subjected to selection by neomycin for four months. The characteristic cellular morphology is maintained throughout this prolonged period of culture.

The preliminary demonstration of the success of the infection of these neurotropic cells and the maintenance of a satisfactory expression of the marker gene carried by the FOCH29 vector led to the continuation of the work in the direction of therapy : the construction of the vector FOCH29-PLP was then achieved. In fact, this gene is defective in patients suffering from the Pelizaeus-Metzbacher disease (Saugier-Veber et al., 1994). The therapeutic advantage of the retroviral transfer of a normal version of the gene into the glial progenitors responsible for the synthesis of the myelin of the central nervous system, or into genetically manipulated Schwann cells is evaluated in mouse models of the human disease (Jimpy mouse, Jimpy/MSD (Pham-Dinh et al., 1992) and Rumpshaker). We are currently monitoring the in vivo fate of genetically manipulated MSC80 cells, after stereotaxic reimplantation in the brain.

The construction FOCH29-PLP uses the endogenous promoter of the PLP gene; this latter is also evaluated on a delU3 version of FOCH29, in which the transcription of the PLP gene is governed by its own promoter.

e-   FIBROBLASTS
        1°- of murine origin
        2°- of canine origin
        3°- of non-human primate origin
        4°- of human origin
f-   CELLS OF HEMATOPOIETIC STROMA
        1°- of murine origin, in the line (line MS5) used for the
long-term cultures of hematopoietic stem cells with expression over

2°- of canine origin on primary cultures of fetal medullary fibroblasts established in the laboratory.

3°- of non-human primate origin

4°- of human origin

g- ENDOTHELIAL CELLS

h- MESENCHYMATOUS CELLS

i- MESOTHELIAL CELLS

j- KERATINOCYTES

k- HEPATOCYTES

l- LINES of human origin

1°- of T LYMPHOCYTES : JURKATT

2°- of NK CELLS : YT2C2

3°- of MONOCYTES-MACROPHAGES : U937

4°- ERYTHRO-MEGAKARYOCYTIC : K562

These four cell lines were infected with a viral supernatant either in a single cycle or in four cycles of infection (two cycles separated by an eight hour interval on two consecutive days). Sixteen hours after the infection the cells are subjected to selection by neomycin at various concentrations : 0.3 mg/ml; 0.5 mg/ml and 1 mg/ml.

A labelling assay with tritiated thymidine was performed one week after the infection (i.e. after five days of selection); this assay proved to be substantially positive for the lines with rapid growth : Jurkatt and K562 whatever the concentration of neomycin used.

After prolonged selection by neomycin during three weeks, the comparative analysis of cellular viability (cells in suspension) between the wells selected at different concentrations and the control wells without selection shows that a considerable percentage of actually transduced cells on which resistance to neomycin was conferred :

T CELL TUMOR LINE JURKATT : 75 to 80%

NK CELLS YT2C2 30 to 50%

MONOCYTES-MACROPHAGES U937 : 40 to 60%

ERYTHRO-MEGAKARYOCYTIC K562 : 75 to 80%

IN VIVO MODEL OF RETROVIRAL TRANSFER APPLICATION TO BLADDER CANCER

Since the bladder is a hollow organ accessible by means of a simple endourethral probe, the forms of administration and the efficiency of gene transfer by direct endovesicular instillation in vivo were evaluated point by point. This evaluation involves several lines of parameters :

1° - Efficiency of retroviral transfer of the vesicular urothelium by in vivo endovesicular inoculation of viral supernatants transporting "reporter" constructions.

2° - Harmlessness of mechanical operations and the transduction of the epithelial cells

3° - Absence of systemic diffusion of the viral particles

4° - Preferential infection of the tumor cells compared with the healthy epithelium

In fact, the superficial bladder tumors, developed at the expense of the vesicular urothelium in 90% of the cases show a progression marked essentially by a tendency to recurrence after complete excision by endoscopic surgery. A group at high risk of recurrence and progression may be defined and includes the tumors of stage pT1, multifocal tumors of stage pTa, in situ carcinomas or the associated dysplasias, and the multiple tumoral recurrences.

In this group, non-specific chemotherapy and immunotherapy by the endovesicular route are used as prophylactic treatments of recurrences after endoscopic surgery. However, the endovesicular treatments currently available have proved incapable of eradicating the multiple tumoral recurrences, and even in certain cases the progression of the tumors. Original therapeutic protocols based on the transfer of genes inhibitory of tumoral invasion (TIMP gene, construction FOCH29) previously mentioned or capable of stimulating the endogenous immune response (constructions FOCH29-IL12, FOCH29-IFN-gamma previously cited), or even interfering with the transduction of the enhancer signals of proliferation in the tumor cells must hence be suggested.

PERSISTENCE OF PACKAGED DEFECTIVE VIRAL PARTICLES IN BIOLOGICAL FLUIDS

In this context the infectivity of the virions after incubation at

or non-filtered) volume by volume was analyzed at sequential time intervals as follows : 5 min; 10 min; 15 min; 20 min; 30 min; 45 min; 60 min; 75 min; 90 min; 120 min; 150 min; 180 min. It was possible to demonstrate by using the beta-gal marker gene with a nuclear localization signal that the viral titers did not vary during the first three hours; the relevance of an approach by in vivo endovesicular instillation associated with water intake restriction is confirmed.

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CLAIMS

1.   Recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence inserted in said vector characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence given in Figure 1A and nucleotide 1527 of the sequence given in Figure 1C, said control fragment having a 5' LTR sequence included between nucleotides 7842 and 144, a PBS site starting at nucleotide 145 of the sequence given in Figure 1A, and a packaging sequence included in the sequence of 250 nucleotides following the end of the LTR sequence, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

2.   Recombinant vector for the cloning and/or expression and/or transfer of an exogenous nucleotide sequence inserted in said vector characterized in that it comprises a control sequence contained in a fragment situated approximately between nucleotide 7702 of the sequence in Figure 1A and nucleotide 310 of the sequence shown in Figure 1D said control sequence having a 5' LTR sequence included between nucleotides 7842 and 144 of Figure 1A, a PBS site starting at nucleotide 145 of Figure 1A, a packaging sequence comprised in the sequence of 250 nucleotides following the end of the 5' LTR sequence, and a 3' LTR sequence included between nucleotide 7842 of the sequence given in Figure 1C and nucleotide 144 of the sequence given in Figure 1D, said control sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

3.   Recombinant vector according to Claim 1 or Claim 2, characterized in that it additionally comprises all or part of the gag sequence situated between nucleotides 619 and 2245 of the sequence shown in Figure 5, in particular the sequence comprised between nucleotides 619 and 1527 of the sequence shown in Figure 5.

4.   Recombinant vector according to Claim 1 or Claim 2, characterized in that the control sequence consists of the fragment situated between nucleotide 7702 of the sequence shown in Figure

1A, and nucleotide 1527 of the sequence shown in Figure 1C.

5.   Recombinant vector according to Claim 2 or Claim 3, characterized in that the control sequence consists of the fragment situated between nucleotide 7702 of the sequence shown in Figure 1A and nucleotide 310 of the sequence shown in Figure 1D.

6.   Recombinant vector according to any one of the Claims 1 to 5, characterized in that it contains in addition at least one polylinker possessing restriction sites unique with respect to the sites contained in the vector.

7.   Recombinant vector according to any one of the Claims 1 to 6, characterized in that it is the plasmid pFOCH29 deposited with the CNCM on 30 June 1993 under No I-1326.

8.   Recombinant vector according to any one of the Claims 1 to 7, characterized in that it comprises in addition a marker gene or part of a marker gene, for example the gene for neomycin resistance.

9.   Recombinant vector according to any one of the Claims 1 to 8, characterized in that the U3 region of the 5' LTR and/or the 3' LTR is deleted at least in part such that the promoter and/or enhancer contained in U3 is (are) at least in part inactivated or modified.

10.   Recombinant vector according to any one of the Claims 1 to 8, characterized in that the U5 region of the 5' LTR and/or the 3' LTR is, at least partially, deleted.

11.   Recombinant vector according to any one of the Claims 1 to 9, characterized in that said control sequence contained in said fragment is replaced by a sequence hybridizing under conditions of high stringency with said control sequence or is replaced by a sequence having a nucleotide homology of at least 95% with said control sequence or of at least 85% with the control sequence.

12.   Recombinant vector according to any one of the Claims 1 to 11, characterized in that it contains the gag and pol sequences of the FB29 strain.

13.   Recombinant vector according to any one of the Claims 1 to 11, characterized in that the exogenous nucleotide sequence is under the control of an exogenous promoter.

14.   Recombinant vector according to any one of the Claims 1 to 12, characterized in that it is introduced in a packaging line.

15. Recombinant vector according to any one of the Claims 1 to 13, characterized in that it is packaged in the psi-CRIP line

16. Recombinant vector according to any one of the Claims 1 to 13, characterized in that it is packaged in the psi-CRE line.

17. Recombinant vector according to any one of the Claims 1 to 15, characterized in that it contains several exogenous sequences.

18. Recombinant vector according to any one of the Claims 1 to 16, characterized in that at least one exogenous sequence is inserted in the LTR sequence.

19. Recombinant vector according to any one of the Claims 1 to 17, characterized in that the exogenous sequence is a sequence of therapeutic importance.

20. Recombinant vector according to any one of the Claims 1 to 17, characterized in that the exogenous sequence codes for an antigen or for an antigenic determinant.

21. Recombinant vector according to any one of the Claims 1 to 19, characterized in that the exogenous sequence is a genomic DNA sequence or a cDNA sequence or a RNA sequence.

22. Prokaryotic or eukaryotic recombinant cell, characterized in that it is modifed by a recombinant vector according to any one of the Claims 1 to 20, in particular the cells of species lacking endogenous retroviruses.

23. Recombinant cell according to Claim 21, characterized in that it is a mammalian cell, in particular a human cell.

24. Recombinant cell according to Claims 22, characterized in that it is a hematopoietic cell, in particular a hematopoietic precursor or a lymphomyeloid totipotent stem cell.

25. Recombinant cell according to Claim 22, characterized in that it is a nerve cell, in particular a neuronal or glial cell, a fibroblast, a hepatic, cutaneous or muscle cell, T or B lymphocytes or other mediator of cellular immunity, tumor cells, medullary stroma, endothelial and mesenchymatous cells.

26. Method for the ex vivo or in vivo transfection of eukaryotic cells, characterized in that the eukaryotic cells are infected by a recombinant retroviral vector according to any one of

ABSTRACT

RETROVIRAL VECTOR FOR THE TRANSFER AND EXPRESSION OF GENES FOR THERAPEUTIC PURPOSES IN EUKARYOTIC CELLS

The invention relates to a recombinantvector for the cloning and/or expression and/or transfer ofan exogenous nucleotide sequence characterized in that it consists of any sequence contained in the ClaI-PvuII fragment comprising nucleotides 7702 to 1527 of the sequence given in Figure 1 and comprising the LTR sequence included between nucleotides 7842 and 144, the PBS site starting at nucleotides 145, the packaging sequence included in the sequences of 250 nucleotides following the end of the LTR sequence, the said sequence being capable of controlling the cloning and/or expression and/or transfer of the exogenous sequence whatever its transcriptional orientation with respect to the transcriptional orientation of the virus.

It relates to the use of this vector for the transfer and/or cloning and/or expression of genes, in particular in the context of gene therapy.

EcoRI

REFMLVCGD.ENV

```
env
AGT GAATTC CGATTAG TTCAATTTGT TAAAGACAGG ATCTCAGTAG 7740
                                             3'bsr U3  IR
TCCAGGCTTT AGTCCTGACT CAACAATACC ACCAGCTAAA ACCACTAGAA TACGAGCCAC 7800
AATAAATAAA AGATTTTATT TAGTTTCCAG AAAAGGGGG GAATGAAAGA CCCCACCAAA 7860
        LTR
TTGCTTAGCC TGATAGCCGC AGTAACGCCA TTTTGCAAGG CATGGAAAAA TACCAAACCA 7920
AGAATAGAGA AGTTCAGATG AAGGGCGGGT ACACGAAAAC AGCTAACGTT GGGCCAAACA 7980
GGATATCTGC GGTGAGCAGT TTCGGCCCCG GCCCGGGGCC AAGAACAGAT GGTCACCGGG 8040
GTTCGGCCCC GGCCCGGGGC CAAGAACAGA TGTCCCCCAG ATATGGCCCA ACCCTCAGCA 8100
GTTTCTTAAG ACCCATCAGA TGTTTCCAGG CTCCCCCAAG GACCTGAAAT GACCCTGTGC 8160
CTTATTTGAA TTAACCAATC AGCCTGCTTC TCGGCTTCTGT TCGCGCGCTT CTGCTTCCCG 8220
 R                              Hg   V3   KpnI
AGCTCTATAA AAGAGCTCAC AACCCCTCAC
                        SmaI
GGGCCAGTCC TCCGATAGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AATCCTCTTG 60
       V5                              5'fg3
CTGTTGCATC CGACTCGTGG TCTCGGTGTT CCTTGGGAGG GTCTCCTCAG AGTGATTGAC 120
       IR           Sb
TACCCGTCTC GGGGGTCTTT CATTTGGGGG CTCGTCCGGG ATCTGGAGAC CCCTGCCCAG 180
                               SnI
GGACCACCGA CCCACCACCG GGAGGTAAGC TGGCCAGCAA TGTTTCTGTG TCTGTCCATT 240
                                         SpI
GTCCCTGTGTC TTTGATTGAT TTTATGCGCC TGTGTCTGTA CTAGTTGGCC GACTAGATTG 300
```

71893/94

BamHI
                                                          →PR75 GCSA
GTATCTGGGG GATCCGTGGT GGAACTGACG AGTTCGAGAC ACCCGGCGGC AACCCTGGGA 360
GACGTCCCAG GGACTTCGGG GGCCATTTTT GTGGCCCGGC CAGAGTCCAA CCATCCCGAT 420
CGTTTTGGAC TCTTTGGTGC ACCCCCCTTA GAGGAGGGGT ATGTGGTTCT GGTAGGAGAC 480
AGAGGGCTAA AACGGTTCC GCCCCGTCT GAGTTTTTGC TTTCGGTTTG GAACCGAAGC 540
CGGCGCGGC GTCTGTGTG CTGCAGCATC GTTCTGTGTT GTCTCTGTTT GACTGTTTTT 600
CTGTATTTGT CTGAAAACAT GGGCCAGGCT GTTACCACCC CCTTAAGTTT GACTTTAGAC 660
CACTGGAAGG ATGTCGAACG GACAGCCCAC AACCTGTCGG TAGAGGTTAG AAAAGGCGC 720
TGGGTTACAT TCTGCTCTGC AGAATGGCCA ACCTTCAACG TCGGATGGCC ACGAGACGGC 780
ACTTTTAACC CAGACATTAT TACACAGGTT AAGATCAAGG TCTTCTCACC TGGCCCACAT 840
GGACATCCGG ATCAGGTCCC CTACATCGTG ACCTGGGAAG CTATAGCAGT AGACCCCCCT 900
CCCTGGGTCA GACCCTTCGT GCACCCTAAA CCTCCCCTCT CTCTCCCCC TTCAGCCCCC 960
TCTCTCCCAC CTGAACCCCC ACTCTCGACC CCGCCCCAGT CCTCCCCTCTA TCCGGCTCTC 1020
ACTTCTCCTT TAAACACCAA ACCTAGGCCT CAAGTCCTTC CTGATAGCGG AGGACCACTC 1080

FIGURE 1b

ATTGATCTAC TCACGGAGGA CCCTCCGCCT TACCGGGACC CAGGGCCACC CTCTCCTGAC 1140

GGGAACGGCG ATAGCGGAGA AGTGGCCCCT ACAGAAGGAG CCCCTGACCC TTCCCCAATG 1200

p12 GTATCCCGCC TGCGGGGAAG AAAGAACCC CCCGTGGCGG ATTCTACTAC CTCTCAGGCG 1260
→CAp30
TTCCCCTTC GCCTGGGAGG GAATGGACAG TATCAATACT GGCCATTTTC CTCCTCTGAC 1320

CTCTATAACT GGAAAAATAA CAACCCCTCT TTCTCCGAGG ACCCAGCTAA ATTGACAGCT 1380

TTGATCGAGT CGGTTCTTCCT TACTCATCAG CCCACTTGGG ATGACTGCCA ACAGCTATTA 1440
debut FMS15 1447. 1468
GGGACCCCTGC TGACGGGAGA AGAAAAACAGV CGAGTGCTCC TAGAGGCCCG AAAGGCGGTT 1500

CGAGGGGAGG ACGGACGCCC AACTCAG Sal I
            Cla   GGGAATCCTCTAGAGAGTCGACCTGCAGGCAATGCAAGCT
                   BamH I   Xba I   Sal I   Pst I   Hind II
                                      Hinc II   Sph I
                                      Acc I

            CAGATCCAAT TCGATTAG TTCAATTTGT TAAAGACAGG ATCTCAGTAG 7740

env TCCAGGCTTT AGTCCTGACT CAACAATACC ACCAGCTAAA ACCACTAGAA TACGAGCCAC 7800
            start U3   LR
AATAAATAAA AGATTTTATT TAGTTTCCAG AAAAGGGGG GAATGAAAGA CCCCACCCAAA 7860

REFMLVCGD.ENV<

7189217

```
TTGCTTAGCC TGATAGCCGC AGTAACGCCA TTTTGCAAGG CATGGAAAAA TACCAAACCA 7920
      LTR 8?
AGAATAGAGA AGTTCAGATC AAGGGGGGGT ACACGAAAAC AGCTAACGTT GGGCCAAACA 7980

GGATATCTGC GGTGAGCAGT TTCGGCCCCG GCCCGGGGCC AAGAACAGAT GGTCACCGCG 8040
                                                      LTR + LTR
GTTCGGCCCC GGCCCGGGGC CAAGAACAGA TGGTCCCCAG ATATGGGCCA ACCCTCAGCA 8100

GTTTCTTAAG ACCCATCAGA TGTTTCCAGG CTCCCCCAAG GACCTGAAAT TACCCCTGTGC 8160

CTTATTTGAA TTAACCAATC AGCCTGCTTC TCGCTTCTGT TCGCGCGCTT CTGCTTCCCG 8220

AGCTCTATAA AAGAGCTCAC AACCCCTCAC
                      U3
    R
GGGCCCAGTCC TCCGATAGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AATCCCTCTTG 60
    U5                          SmaI
CTGTTGCAATC CGACTCGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCAG AGTGATTGAC 120
       TR              PBS
TACCCGTCTC GGGGTCTTT CATTTGGGGG CTCGTTCCGGG ATCTGGAGAC CCCTGCCCAG 180
         SD
GGACCACCGA CCCACCACCG GGAGGTAAGC TGGCCAGCAA TTGTTCTGTG TCTGTCCATT 240
                                           SpeI
GTCCTGTGTC TTTGATTGAT TTTATGGGCC TGTGTCTGTA CTAGTGGCC GACTAGATTG 300
    BamHI
GTATCTGGCG GATC  T  -> pUC19
```

FIGURE 1d

FIGURE 2 A

FOCH 29
(5664 pb)

pUC 19

pbs psi gag

Eco R1(1)
Esp I/7862/(166)
Eco RV/7982(286)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam HII/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)
polylinker(2088)
Esp I(2283)
Eco RV(2403)
Bss HII(2624)
Kpn I(2707)
Bgl II(2991)
Spe I(2965)

U3
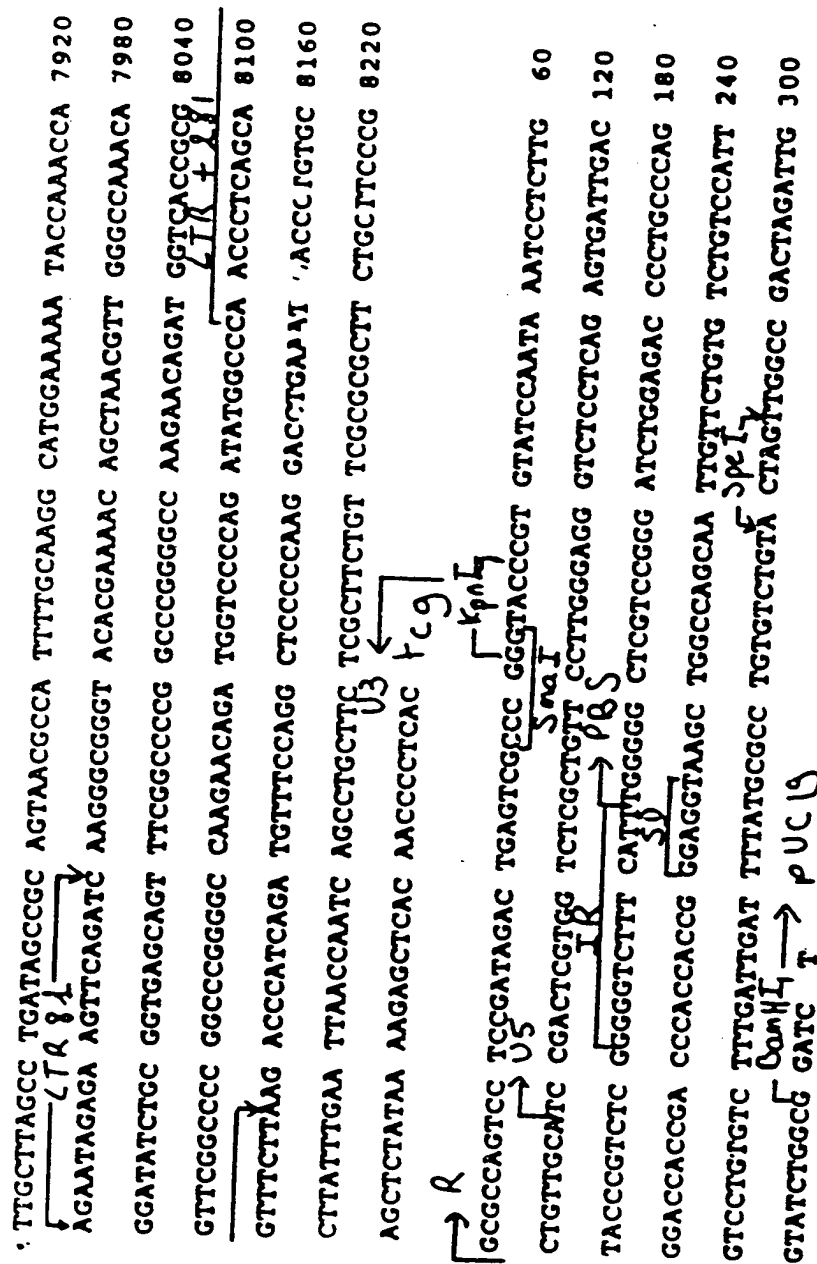R
U5

polylinker
Bam HI : 2088
Xba I : 2094
Sal I : 2100
Pst I : 2106
Sph I : 2112

FIGURE 2B

FIGURE 3

pUC19
(ATCC 37254)
2.686 base pairs
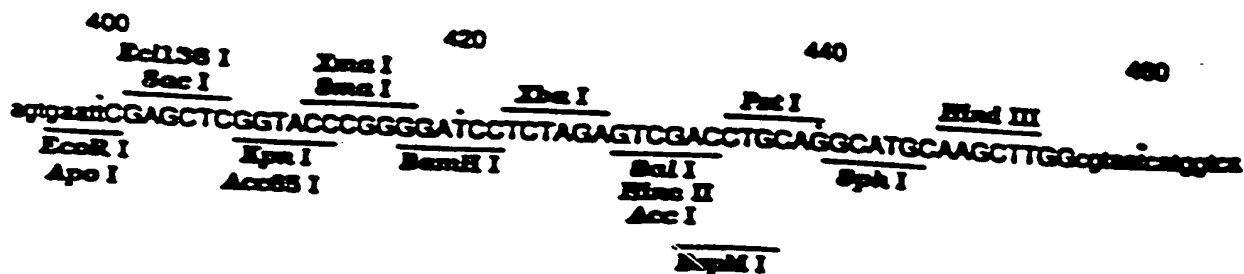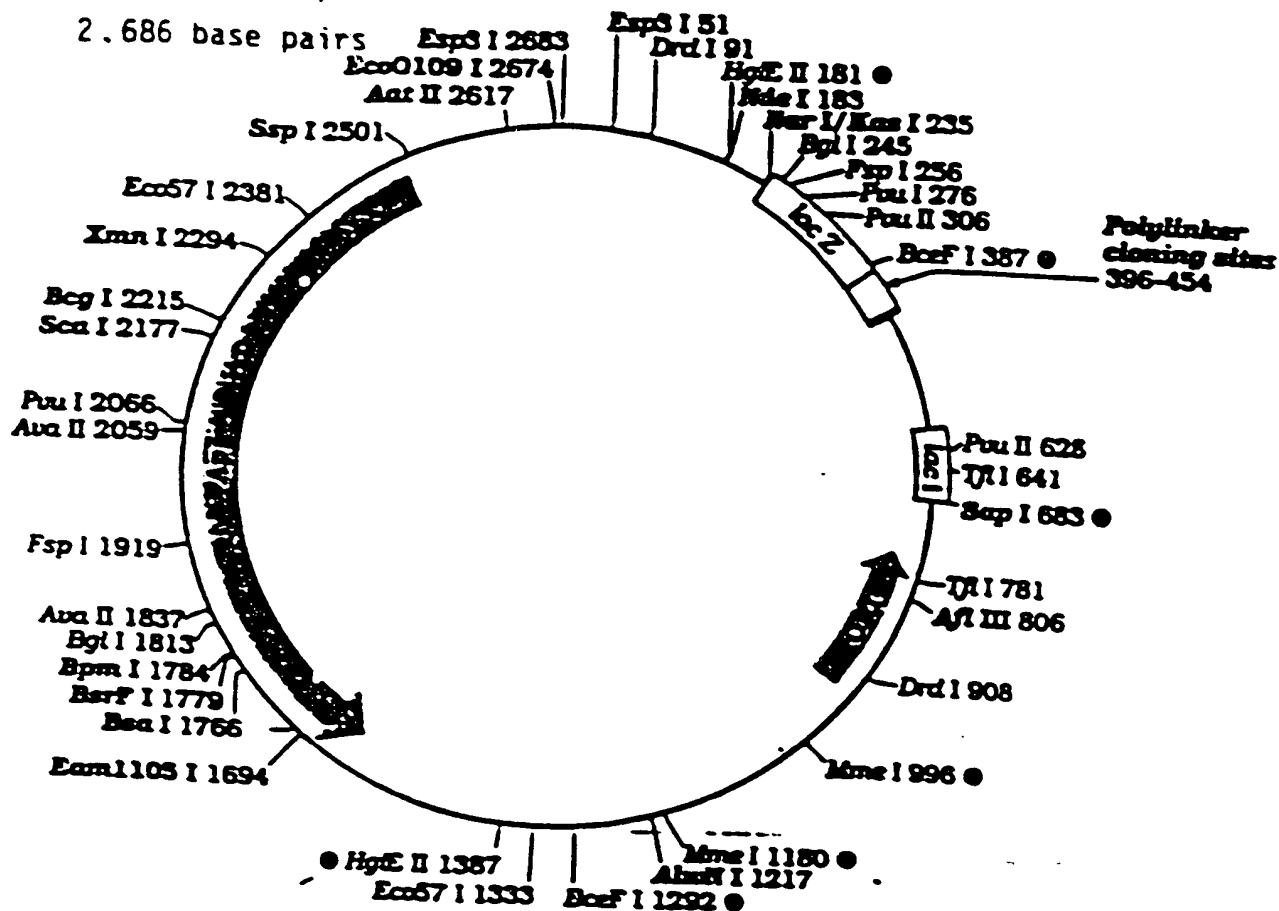
FIGURE 4

F.

KpnI

GCGCCAGTCC TCCGATAGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AATCCTCTTG 60

U5

SmaI

CTGTTGCATC CGACTCGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCAG AGTGATTGAC 120

TR PBS

TACCCGTCTC GGGGGTCTTT CATTTGGGGG CTCGTCCGGG ATCTGGAGAC CCCTGCCCAG 180

SD

GGACCACCGA CCCACCACCG GGAGGTAAGC TGGCCAGCAA TTGTTCTGTG TCTGTCCATT 240

SpeI

GTCCTGTGTC TTTGATTGAT TTTATGCGCC TGTGTCTGTA CTAGTTGGCC GACTAGATTG 300

BamHI Pr75 GCSA

GTATCTGGCG GATCCGTGGT GGAACTGACG AGTTCGAGAC ACCCGGCCGC AACCCTGGGA 360

GACGTCCCAG GGACTTCGGG GGCCATTTTT GTGGCCCGGC CAGAGTCCAA CCATCCCGAT 420

CGTTTTGGAC TCTTTGGTGC ACCCCCCTTA GAGGAGGGGT ATGTGGTTCT GGTAGGAGAC 480

AGAGGGCTAA AACGGTTTCC GCCCCCGTCT GAGTTTTTGC TTTCGGTTTG GAACCGAAGC 540

PstI

CGCGCCGCGC GTCTTGTCTG CTGCAGCATC GTTCTGTGTT GTCTCTGTTT GACTGTTTTT 600

Gug

CTGTATTTGT CTGAAAACAT GGGCCAGGCT GTTACCACCC CCTTAAGTTT GACTTTAGAC 660

REPH LVCGD.GAG MAp15

CACTGGAAGG ATGTCGAACG GACAGCCCAC AACCTGTCGG TAGAGGTTAG AAAAAGGCGC 720

TGGGTTACAT TCTGCTCTGC AGAATGGCCA ACCTTCAACG TCGGATGGCC ACGAGACGGC 780

ACTTTTAACC CAGACATTAT TACACAGGTT AAGATCAAGG TCTTCTCACC TGGCCCACAT 840

GGACATCCGG ATCAGGTCCC CTACATCGTG ACCTGGGAAG CTATAGCAGT AGACCCCCCT 900

CCCTCGGTCA GACCCTTCGT GCACCCTAAA CCTCCCCTCT CTCTTCCCCC TTCAGCCCCC 960

MAp15 pp12

TCTCTCCCAC CTGAACCCCC ACTCTCGACC CCGCCCCAGT CCTCCCTCTA TCCGGCTCTC 1020

StuI

ACTTCTCCTT TAAACACCAA ACCTAGGCCT CAAGTCCTTC CTGATAGCGG AGGACCACTC 1080

ATTGATCTAC TCACGGAGGA CCCTCCGCCT TACCGGGACC CAGGGCCACC CTCTCCTGAC 1140

GGGAACGGCG ATAGCGGAGA AGTGGCCCCT ACAGAAGGAG CCCCTGACCC TTCCCCAATG 1200

GTATCCCGCC TGCGGGGAAG AAAAGAACCC CCCGTGGCGG ATTCTACTAC CTCTCAGGCG 1260

p12 CAp30

TTCCCCCTTC GCCTGGGAGG GAATGGACAG TATCAATACT GGCCATTTTC CTCCTCTGAC 1320

CTCTATAACT GGAAAAATAA CAACCCCTCT TTCTCCGAGG ACCCAGCTAA ATTGACAGCT 1380

TTGATCGAGT CCGTTCTCCT TACTCATCAG CCCACTTGGG ATGACTGCCA ACAGCTATTA 1440

début FMS15 1447-1468

GGGACCCTGC TGACGGGAGA AGAAAAACAG CGAGTGCTCC TAGAGGCCCG AAAGGCGGTT 1500

PvuII

CGAGGGGAGG ACGGACGCCC AACTCAGCTG CCCAATGACA TTAATGATGC TTTTCCCTTG 1560
(1527) N+B+

GAACGTCCCG ACTGGGACTA CAACACCCAA CGAGGTAGGA ACCACCTAGT CCACTATCGC 1620

CAGTTGCTCC TAGCGGGTCT CCAAAACGCG GCAGAAGCC CCACCAATTT GGCCAAGGTA 1680

AAAGGGATAA CCCAGGGACC TAATGAGTCT CCCTCAGCCT TTTTAGAGAG ACTCAAGGAG 1740

GCCTATCGCA GATACACTCC TTATGACCCT GAGGACCCAG GGCAAGAAAC CAATGTGGCC 1800

FIGURE 5A

ATGTCATTCA TCTGGCAGTC CGCCCCGGAT ATCGGGCGAA AGTTAGAGCG GTTAGAAGAT 1860

TTGAAGAGTA AGACCTTAGG AGACTTAGTG AGGGAAGCTG AAAAGATCTT TAATAAACGA 1920
                                                 BglII

GAAACCCCGG AAGAAAGAGA GGAACGTATT AGGAGAGAAA CAGAGGAAAA GGAAGAACGC 1980

CGTAGGGCAG AGGATGTGCA GAGAGAGAAG GAGAGGGACC GCAGAAGACA TAGAGAAATG 2040
      CAp30 ←——→NCp10

AGTAAGTTGC TGGCTACTGT CGTTAGCGGG CAGAGACAGG ATAGACAGGG AGGAGAGCGA 2100

AGGAGGCCCC AACTCGACCA CGACCAGTGT GCCTACTGCA AAGAAAGGG ACATTGGGCT 2160

AGAGATTGCC CCAAGAAGCC AAGAGGACCC CGGGGACCAC GACCCCAGGC CTCCCTCCTG 2220
         gag ←——→pol   →PR

ACCTTAGACG ATTAGGGAGG TCAGGGTCAG GAGCCCCCCC CTGAACCCAG ATAACCCTC 2280
>REFMLVCGD POL
  REFMLVCGD.GAG<

AGAGTCGGGG GGCAACCCGT CACCTTCCTA GTGGATACTG GGCCCAACA CTCCGTGCTG 2340

ACCCAAAATC CTGGACCCCT AAGTGACAAG TCTGCCTGGG TCCAAGGGGC TACTGGAGGG 2400

AAGCGGTATC GCTGGACCAC GGATCGCCGA GTGCACCTAG CCACCGGTAA GGTCACCCAT 2460

TCTTTCCTCC ATGTACCAGA TTGCCCCTAT CCTCTGCTAG GAAGAGATTT GCTGACTAAA 2520

CTAAAAGCCC AAATTCACTT TGAGGGATCA GGAGCTCAGG TTGTGGGACC AATGGGACAG 2580
        PR ←——→RT

CCCCTGCAAG TGCTGACCCT AAACATAGAA GATGAGTATC GGCTACATGA GACCTCAAAA 2640

GGGCCAGATG TGCCTCTAGG GTCCACATGG CTCTCTGATT TTCCCCAGGC CTGGGCAGAA 2700

ACCGGGGGCA TGGGGCTGGC CGTTCGCCAA GCTCCTCTGA TCATACCTCT GAAGGCAACC 2760
                                                   BclI

TCTACCCCCG TGTCCATAAA ACAATACCCC ATGTCACAAG AAGCCAGACT GGGGATCAAG 2820

CCCCACATAC AGAGACTGCT GGATCAGGGA ATTCGGTAC CCTGCCAGTC CCCCTGGAAC 2880
                                EcoRI

ACGCCCCTGC TACCCGTTAA GAAACCGGGG ACTAATGATT ATAGGCCTGT CCAGGATCTG 2940

AGAGAAGTCA ACAAGCGGGT GGAAGACATC CACCCCACCG TGCCCAACCC TTACAACCTC 3000

TTGAGCGGGC TCCCACCGTC CCACCAGTGG TACACTGTGC TTGACTTAAA AGATGCTTTT 3060

TTCTGCCTGA GACTCCACCC CACCAGTCAG TCTCTCTTCG CCTTTGAGTG GAGAGATCCA 3120

GAGATGGGAA TCTCAGGACA ATTAACCTGG ACCAGACTCC CGCAGGGTTT CAAAAACAGT 3180

CCCACCCTGT TTGATGAAGC CCTGCACAGG GACCTCGCAG ACTTCCGGAT CCAGCACCCA 3240

GACCTGATTC TGCTCCAGTA TGTAGATGAC TTACTGCTGG CCGCCACTTC TGAGCTTGAC 3300

TGTCAACAAG GTACGCGGGC CCTGTTACAA ACCCTAGGG ACCTCGGATA TCGGGCCTCG 3360

GCCAAGAAAG CCCAAATTTG CCAGAAACAG GTCAAGTATC TGGGGTATCT TCTAAAGAG 3420

GGTCAGAGAT GGCTGACTGA GGCCAGAAAA GAGACTGTGA TGGGGCAGCC TACTCCGAAG 3480

ACCCCTCGAC AACTAAGGGA GTTCCTAGGG ACGGCAGGCT TCTGTCGCCT CAGGATCCCT 3540

GGGTTTGCAG AAATGGCAGC CCCCTTGTAC CCTCTCACCA AAACGGGGAC TCTGTTTGAG 3600

TGGGGCCCAG ACCAGCAAAA GGCCTACCAA GAGATCAAGC AGGCTCTCTT AACTGCCCCT 3660

```
GCCCTGGGAT TGCCAGACTT GACTAAGCCC TTCGAACTTT TTGTTGACGA GAAGCAGGGC 3720
TACGCCAAAG GTGTCCTAAC GCAAAAACTG GGGCCTTGGC GTCGGCCGGT GGCCTACCTG 3780
TCCAAAAAGC TAGACCCAGT GGCAGCTGGG TGGCCCCCTT GCCTACGGAT GGTAGCAGCC 3840
ATCGCCGTTC TGACCAAAGA CGCTGGCAAG CTCACCATGG GACAGCCACT AGTCATTCTG 3900
GCCCCCCATG CAGTAGAGGC ACTAGTTAAG CAACCCCCTG ATCGCTGGCT CTCCAACGCC 3960
CGAATGACCC ACTACCAGGC TCTGCTTCTG GACACGGACC GAGTCCAGTT CGGACCAATA 4020
GTGGCCCTAA ACCCAGCTAC GCTGCTCCCT CTACCTGAGG AGGGGCTGCA ACATGACTGC 4080
CTTGACATCT TGGCTGAAGC CCACGGAACT AGACCAGATC TTACGGACCA GCCTCTCCCA 4140
GACGCTGACC ACACCTGGTA CACAGATGGG AGCAGCTTCC TGCAAGAGGG GCAGCGCAAG 4200
GCCGGAGCAG CAGTAACCAC CGAGACCGAG GTAGTCTGGG CCAAAGCACT GCCAGCCGGG 4260
ACATCGGCCC AAAGAGCTGA GTTGATAGCG CTCACCCAAG CCTTAAAAAT GGCAGAAGGT 4320
AAGAAGCTGA ATGTTTACAC CGATAGCCGT TATGCTTTTG CCACTGCCCA TATTCACGGA 4380
GAAATATATA GAAGGCGCGG GTTGCTCACA TCAGAAGGAA AAGAAATCAA AAATAAGGAC 4440
GAGATCTTGG CCCTACTGAA GGCTCTCTTC CTGCCCAAAA GACTTAGCAT AATTCATTGC 4500
CCGGGACATC AGAAGGGAAA CCGCGCGGAG GCAAGGGGCA ACAGGATGGC CGACCAAGCG 4560
GCCCGAGAAG TAGCCACTAG AGAAACTCCA GAGACTTCCA CACTTCTGAT AGAAAATTCA 4620
GCCCCCTATA CTCATGAACA TTTTCACTAT ACGGTGACTG ACATAAAAGA TCTGACTAAA 4680
CTAGGGGCCA CTTATGACGA TGCAAAGAAG TGTTGGGTTT ATCAGGGAAA GCCTGTAATG 4740
CCTGATCAAT TCACCTTTGA ACTATTAGAT TTTCTTCATC AATTGACCCA CCTCAGTTTC 4800
TCAAAAACAA AGGCTCTTCT AGAAAGGAAC TACTGTCCTT ATTACATGCT GAACCGGGAT 4860
CGAACGCTCA AAGACATCAC TGAGACTTGC CAAGCCTGTG CACAGGTCAA TGCCAGCAAG 4920
TCTGCCGTCA AACAAGGGAC TAGAGTTCGA GGGCACCGAC CCGGCACCCA CTGGGAAATT 4980
GATTTCACTG AGGTAAAACC TGGCCTGTAT GGGTATAAAT ATCTTTTAGT TTTCATAGAC 5040
ACTTTCTCTG GATGGGTAGA AGCTTTCCCA ACCAAGAAAG AAACTGCCAA AGTTGTAACC 5100
AAGAAGCTAC TAGAAGAAAT CTTCCCCAGA TTCGGCATGC CACAGGTATT GGGAACCGAC 5160
AATGGGCCTG CCTTCGTCTC CAAGGTAAGT CAGACAGTAG CCGATTTACT GGGGGTTGAT 5220
TGGAAACTAC ATTGTGCTTA CAGACCCCAG AGTTCAGGTC AGGTAGAAAG AATGAATAGG 5280
ACAATCAAGG AGACTTTAAC TAAATTGACG CTTGCAACTG GCTCTAGGGA CTGGGTGCTC 5340
CTGCTTCCCC TAGCCCTGTA TCGAGCCCGC AACACGCCGG GCCCCCATGG TCTCACCCCA 5400
```

FIGURE 5C

TATGAAATCT TATATGGGGC ACCCCCGCCC CTTGTAAACT TCCCTGATCC TGACATGGCA 5460

AAGGTTACTC ATAACCCCTC TCTCCAAGCC CATTTACAGG CACTCTACCT GGTCCAGCAC 5520

GAAGTCTGGA GACCGTTGGC GGCAGCTTAC CAAGAACAAC TGGACCGGCC GGTAGTGCCT 5580

CACCCTTTCC GAGTCGGTGA CACAGTGTGG GTCCGCAGAC ACCAAACTAA AAATCTAGAA 5640

CCCCGCTGGA AAGGACCTTA TACCGTCCTA CTGACTACCC CCACCGCTCT CAAAGTGGAC 5700

GGCATTGCAG CGTGGATCCA CGCTGCCCAC GTAAAGGCTG CCGACACCAG GATTGAGCCA 5760

CCATCGGAAT CGACATGGCG TGTTCAACGC TCTCAAAATC CCCTAAAGAT AAGATTGACC 5820
                  >REFMLVCGD.ENV
CGCGGGACCT CCTAATCCCC TTAATTCTCT TCCTGTCTCT CAAAGGGGCC AGATCCGCAG 5880
REFMLVCGD.POL<
CACCCGGCTC CAGCCCTCAC CAGGTCTACA ACATTACCTG GGAAGTGACC AATGGGGATC 5940

GGGAGACAGT ATGGGCAATA TCAGGCAACC ACCCTCTGTG GACTTGGTGG CCAGTCCTCA 6000

CCCCAGATTT GTGTATGTTA GCTCTCAGTG GGCCGCCCCA CTGGGGGCTA GAGTATCAGG 6060

CCCCCTATTC CTCGCCCCCG GGGCCCCCTT GTTGCTCAGG GAGCAGCGGG AACGTTGCAG 6120

GCTGTGCCAG AGACTGCAAC GAGCCCTTGA CCTCCCTCAC CCCTCGGTGC AACACTGCCT 6190

GGAACAGACT TAAGCTGGAC CAGGTAACTC ATAAATCAAG TGAGGGATTT TATGTCTGCC 6240

CCGGGTCACA TCGCCCCCGG GAAGCCAAGT CCTGTGGGGG TCCAGACTCC TTCTACTGTG 6300

CCTCTTGGGG CTGCGAGACA ACCGGTAGAG TATACTGGAA GCCCTCCTCT TCTTGGGACT 6360

ACATCACAGT AGACAACAAT CTCACCTCTA ACCAGGCTGT TCAGGTATGC AAAGACAATA 6420

AGTGGTGCAA TCCCTTGGCT ATCCGGTTTA CAAACGCCGG GAAACAGGTC ACCTCATGGA 6480

CAACTGGACA CTATTGGGGT CTACGTCTTT ATGTCTCTGG ACAGGACCCA GGGCTTACTT 6540

TCGGGATCCG ACTCAGTTAT CAAAATCTAG GACCTCGGAT CCCAATAGGA CCAAACCCCG 6600

TCCTGGCAGA CCAACTTTCG TTCCCGCTAC CTAATCCCCT ACCCAAACCT GCCAAGTCTC 6660

CCCCCGCCTC TAGTTCGACT CCCACATTGA TTTCCCCGTC CCCCACTCCC ACTCAGCCCC 6720

CGCCAGCAGG AACGGGAGAC AGATTACTAA ATCTAGTACA GGGAGCTTAC CAGGCACTCA 6780

ACCTTACCAA CCCTGATAAA ACTCAAGAGT GCTGGTTATG CCTAGTGTCT GGACCCCCCT 6840

ATTACGAGGG GGTTGCCGTC CTAGGTACTT ATTCCAACCA TACCTCTGCC CCAGCTAACT 6900

GCTCCGTGGC CTCCCAACAC AAGCTGACCC TGTCCGAAGT GACTGGACGG GGACTCTGCA 6960

TAGGAACAGT CCCAAAAACT CACCAGGCCC TGTGCAACAC TACCCTTAAG GCAGGCAAAG 7020

GGTCTTACTA TCTAGTTGCC CCCACAGGAA CTATGTGGGC ATGTAACACT GGACTCACTC 7080

CATGCCTATC TGCCACCGTG CTTAATCGCA CCACTGACTA TTGCGTTCTC GTGGAATTAT 7140

FIGURE 5D

```
GGCCCAGGGT CACCTACCAT CCTCCCAGTT ACGTCTATAG CCAGTTTGAA AAATCCCATA 7200

GACATAAAAG AGAACCAGTG TCCTTAACCT TGGCCTTATT ATTAGGTGGG CTAACTATGG 7260

GTGGCATCGC CGCGGGAGTA GGGACAGGAA CTACCGCCCT GGTCGCCACC CAGCAGTTTC 7320

AGCAGCTCCA TGCTGCCGTA CAAGATGATC TCAAAGAAGT CGAAAAGTCA ATTACTAACC 7380

TAGAAAAGTC TCTTACTTCG TTGTCTGAGG TTGTACTGCA GAATCGACGA GGCCTAGACC 7440

TGTTGTTCCT AAAAGAGGGA GGACTGTGTG CTGCCCTAAA AGAAGAATGT TGTTTCTATG 7500

CTGACCATAC AGGCCTAGTA AGAGATAGTA TGGCCAAATT AAGAGAGAGA CTCTCTCAGA 7560

GACAAAAACT ATTTGAGTCG AGCCAAGGAT GGTTCGAAGG ATGGTTTAAC AGATCCCCCT 7620

GGTTTACCAC GTTGATATCC ACCATCATGG GGCCTCTCAT TATACTCCTA CTAATTCTGC 7680

TTTTTGGACC CTGCATTCTT AATCGATTAG TTCAATTTGT TAAAGACAGG ATCTCAGTAG 7740

TCCAGGCTTT AGTCCTGACT CAACAATACC ACCAGCTAAA ACCACTAGAA TACGAGCCAC 7800

AATAAATAAA AGATTTTATT TAGTTTCCAG AAAAAGGGGG GAATGAAAGA CCCCACCAAA 7860

TTGCTTAGCC TGATAGCCGC AGTAACGCCA TTTTGCAAGG CATGGAAAAA TACCAAACCA 7920

AGAATAGAGA AGTTCAGATC AAGGGCGGGT ACACGAAAAC AGCTAACGTT GGGCCAAACA 7980

GGATATCTGC GGTGAGCAGT TTCGGCCCCG GCCCGGGGCC AAGAACAGAT GGTCACCGCG 8040

GTTCGGCCCC GGCCCGGGGC CAAGAACAGA TGGTCCCCAG ATATGGCCCA ACCCTCAGCA 8100

GTTTCTTAAG ACCCATCAGA TGTTTCCAGG CTCCCCCAAG GACCTGAAAT GACCCTGTGC 8160

CTTATTTGAA TTAACCAATC AGCCTGCTTC TCGCTTCTGT TCGCGCGCTT CTGCTTCCCG 8220

AGCTCTATAA AAGAGCTCAC AACCCCTCAC TCGGCGCGCC AGTCCTCCGA TAGACTGAGT 8280

CGCCCGGGTA CCCGTGTATC CAATAAATCC TCTTGCTGTT GCA                  8323
```

Annotations on figure:
- LTR 8₁ (underlined over AGAATAGAGA AGTTCAGATC region, line 7980)
- LTR + 2₄₁ (over GGTCACCGCG region, line 8040)
- U3 ← → R (under region line 8160)

FIGURE 5E

Esp I/7862/(166)
Eco RV/7982(286)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam HI/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)
Eco R1(1)
polylinker(2088)
Kpn I(2367)
Spe I(2615)
Bgl II(2651)

pbs psi gag

U3
R
U5

Foch 29 del U3
(5319 pb)

pUC 19

polylinker :
Bam HI : 2088
Xba I : 2094
Sal I : 2100
Pst I : 2106
Sph I : 2112

FIGURE 6

linker en 5' de U3 :

5' AAT TCA ATG AAA GAC CCC ACC AAA TTG C   3'
3'   GT TAC TTT CTG GGG TGG TTT AAC GAA T 5'

polylinker
Bam HI : 1948
Xba I : 1954
Sal I : 1960
Pst I : 1966
Sph I : 1972

Eco RI(1)

Esp I/7862/(26)

Eco RV/7982(146)

Bss HII/8203/(367)

Kpn I/31/(450)

Spe I/279/(698)

Bam HI/310/(729)

Pst I/560/(979)

Pst I/737/(1156)

pbs   psi   gag

polylinker(1948)

Esp I(2003)

Eco RV(2123)

Bss HII(2344)

Kpn I(2427)

Spe I(2675)

Bgl II(2711)

U3

R U5

U3

R

U5

Foch 29 PL
(5378 pb)

pUC 19

FIGURE 7

Esp I/7862/(166)

Eco RV/7982(286)

Bss HII/8203/(507)

Kpn I/31/(590)

Spe I/279/(838)

Bam HI/310/(869)

pbs psi short gag

Pst I/560/(1119)

Pst I/737/(1296)

polylinker(1592)

Esp I(1787)

Eco RV(1907)

Bss HII(2128)

Kpn I(2211)

Spe I(2459)

Eco RI(1)

U3

R

U5

Foch 29 SG
(5162 pb)

pUC 19

U3

R

U5

Bgl II(2495)

polylinker :
Bam HI : 1592
Xba I : 1598
Sal I : 1604
Pst I : 1610
Sph I : 1616

FIGURE 8

FIGURE 9

FOCH 29-IL12
(8160 pb)

p35 PstI (+188)/EcoRI(1064)
p40 NcoI (+8)/EcoRI(1009)
IRES-EMCV EcoRI (280)/NcoI (847)

FIGURE 10

FOCH 29-NIRIL12
(10284 pb)

p35 PstI (+188)/EcoRI(1064)
p40 NcoI (+8)/EcoRI(1009)
IRES-EMCV EcoRI (280)/NcoI (847)
IRES-Polio KpnI (66)/BalII (627)
NEO BamHI/BamHI

FIGURE 11

FOCH 29-IL12 35IR40N
(10242 pb)

p35 PstI (+188)/EcoRI(1064)
p40 NcoI (+8)/EcoRI(1009)
IRES-EMCV EcoRI (280)/NcoI
(847)
IRES-Pollo KpnI (66)/BalII (627)
NEO BamHI/BamHI

FIGURE 12

FIGURE 13

Foch 29 del U3 nls Lac Z
(8619 pb)

FIGURE 14

Foch 29 del U3 neo
(6819 pb)

PUC 19

U3

pbs psi gag

neo

polylinker(2008)

Eco R1(1)
Esp I/7862/(166)
Eco RV/7982(266)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam HI/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)

Bgl II(4151)
Spe I(4115)
Kpn I(3965)

FIGURE 15

Foch 29 MDR
(5658 pb)

FOCH 29 Xba I blunt/Sal I
MDR Sac I blunt / Xho I

PUC 19

MDR

Eco R1(1)
Esp I/7862/(166)
Eco RV/7982(286)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam I II/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)

pbs psi gag

U3
R U5

Bam HI (2088)
Xba
Sac I

U5 R U3

Bgl II(6991)
Spe I(6955)
Kpn I(6707)
Bss HII(6624)
Eco RV(6403)
Esp I(6344)
Sal I
Xho I

FIGURE 16

FIGURE 17

FIGURE 18

FIGURE 19

FIGURE 20

FIGURE 21

Eco R1(1)

Esp I/7862/(166)
Eco RV/7982(286)
Bss HIII/8203/(507)

Kpn I/31/(590)
Spe I/279/(838)
Bam HI/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)

Bam HI(2088)
Xba I(2094)
Sal I(2100)

U3

pbs psi gag

nls LacZ

FOCH 29 del U3 nls Lac Z HYGRO
(10676 pb)

FOCH BglII
HYGRO Bam HI

pUC 19

Bst YI(8008)

HYGRO

Bst YI(6151)
Spe I(6115)
Kpn I(5867)

Sph I(5806)

FIGURE 22

Foch 29 del U3 HYGRO
(7176 pb)

FOCH BglII
HYG Bam HI

polylinker :
Bam HI : 2088
Xba I : 2094
Sal I : 2100
Pst I : 2106
Sph I : 2112

pUC 19

HYGRO

Eco RI(1)
Esp I/7862/(166)
Eco RV/7982(286)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam HII/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)
polylinker(2088)
Kpn I(2367)
Spe I(2615)
Bst YI(2651)
Bst YI(4508)

U3 R US

pbs psi gag

U3 R US

FIGURE 23

Pure LTR-SD-Del Psi-GAG/POL
(9794pb)

del Psi : Spel 698-PstI704 (init 979)

GAG-POL : PstI 881-HindIII 5204-SmaI 6223

PolyA SV40 : XhoI -EcoRI 884 bp (HpaI-EcoRI)
2666-1782)

Eco RI(1)
Esp I/7862/(26)
Eco RV/7982(146)
Bss HII/8203/(367)
Kpn I/31/(450)
SpeI(698)
PstI(704)
HaeII(864)
PstI(881)
AhaIII(1175)

E829
280
561
720
737
1031

U 3
R
GAG
pol
end

HindIII(5204)
5060 (FÏ329)

SmaI 6079 FÏ329

Esp I/7862/(166)
Eco RV/7982(286)
Bss HII/8203/(507)
Kpn I/31/(590)
Spe I/279/(838)
Bam HI/310/(869)
Pst I/560/(1119)
Pst I/737/(1296)

Bam HI(2088)
Xba I(2094)
SalI(2100)
XbaI(2106)
BamHI(2112)

Eco R1(1)

Foch 29 PLP-nls Lac Z
(11001 pb)

FOCH 29 SalI/SphI-Klenow

+

1° Clone pUC18 :
1-1° Promo PLP AluI/Bam HI (472 bp) +
PLP BamHI/EcoRI fill-in Klenow
1-2° nls Lac Z Bam HI/Bam HI

2°Clone In FOCH as SalI/EcoRI/Kle

BamHI(6072)
SmaI/AluI(5612)
BamHI(5606)

Bgl II(8334)
Spe I(8298)
Kpn I(8050)
Bss HII(7967)
Eco RV(7746)
Esp I(7626)
SphI/Kle(7455)
EcoRI/Kle(7449)

FIGURE 24

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/86   C12N5/10   C12N5/16   C12N5/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Genbank database ,Bethesda, US GenBank ACC. NO. (GBN): Z22761 Bestwick,R. et al. Retroviral expression vector... & PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.85, no.15, 1988, WASHINGTON US pages 5404 - 5408 BESTWICK R.K. ET AL. see the whole document ---	1-9, 11-14, 17-26
Y	Genbank Databank Bethesda GenBank ACC. NO. (GBN): M60088 Yu,X.-F et al. Synthetic plasmid p806 polylinker site. & J.Virol. vol.64, 1990 pages 5688-5693 --- -/--	1-5

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
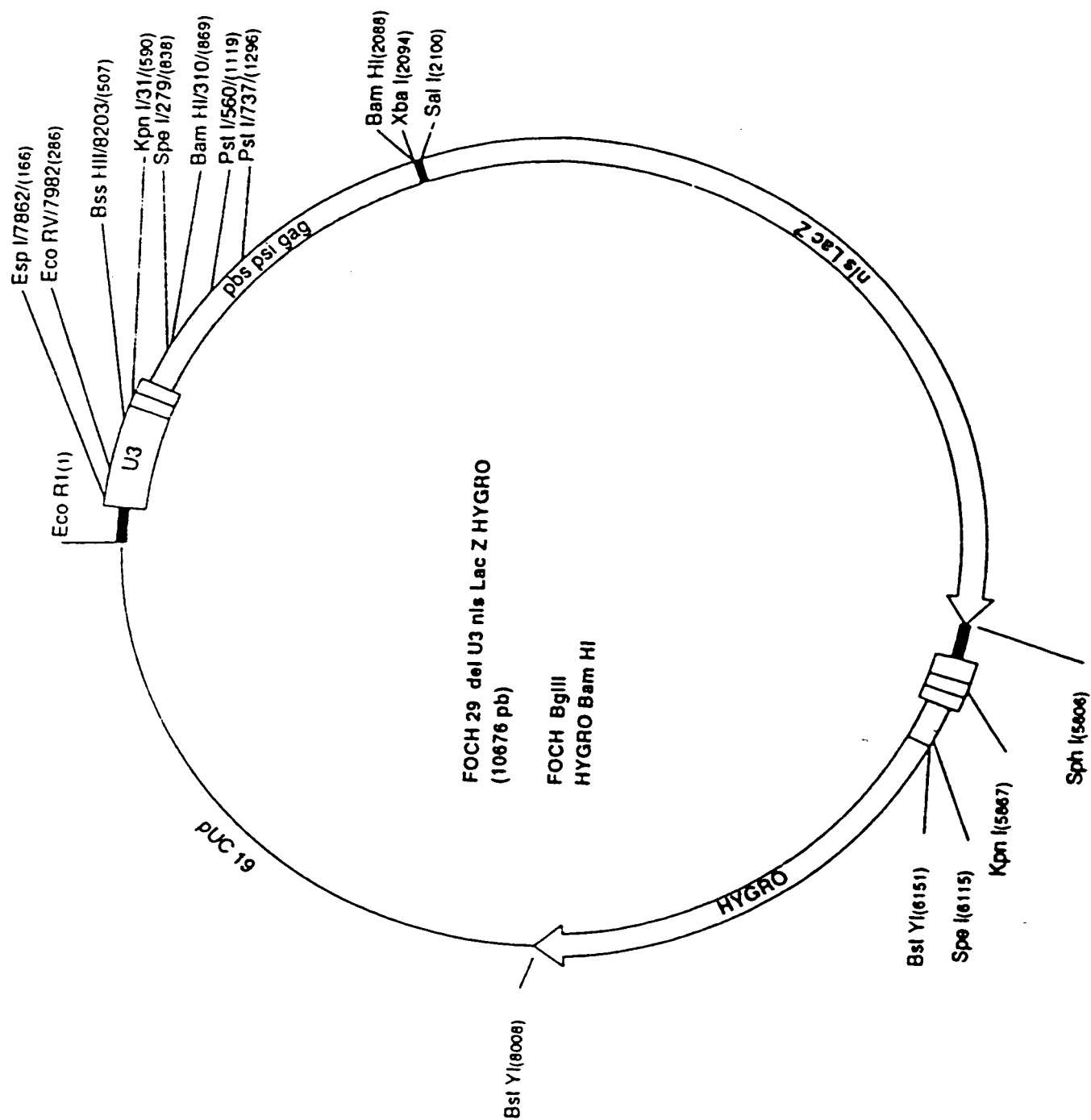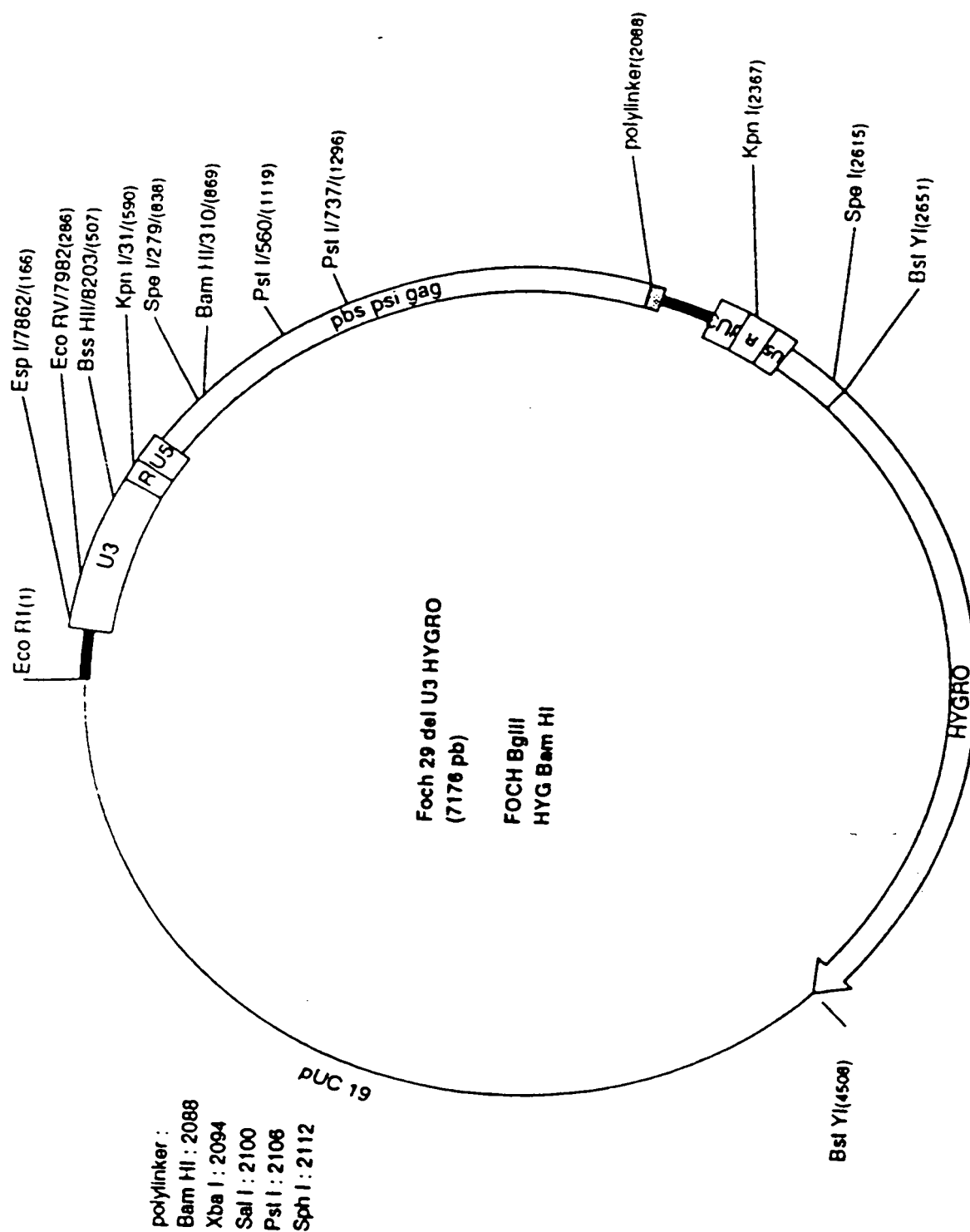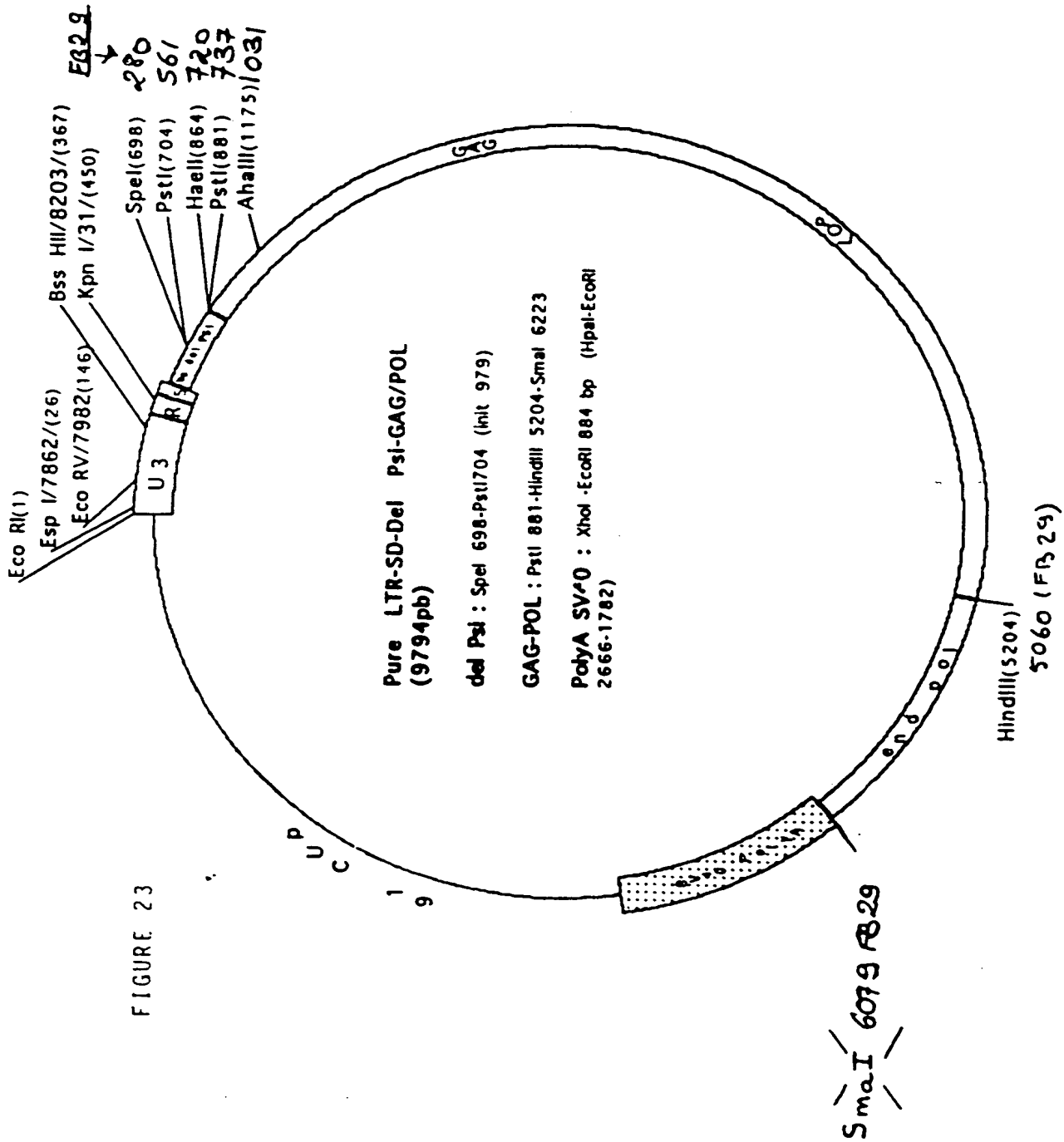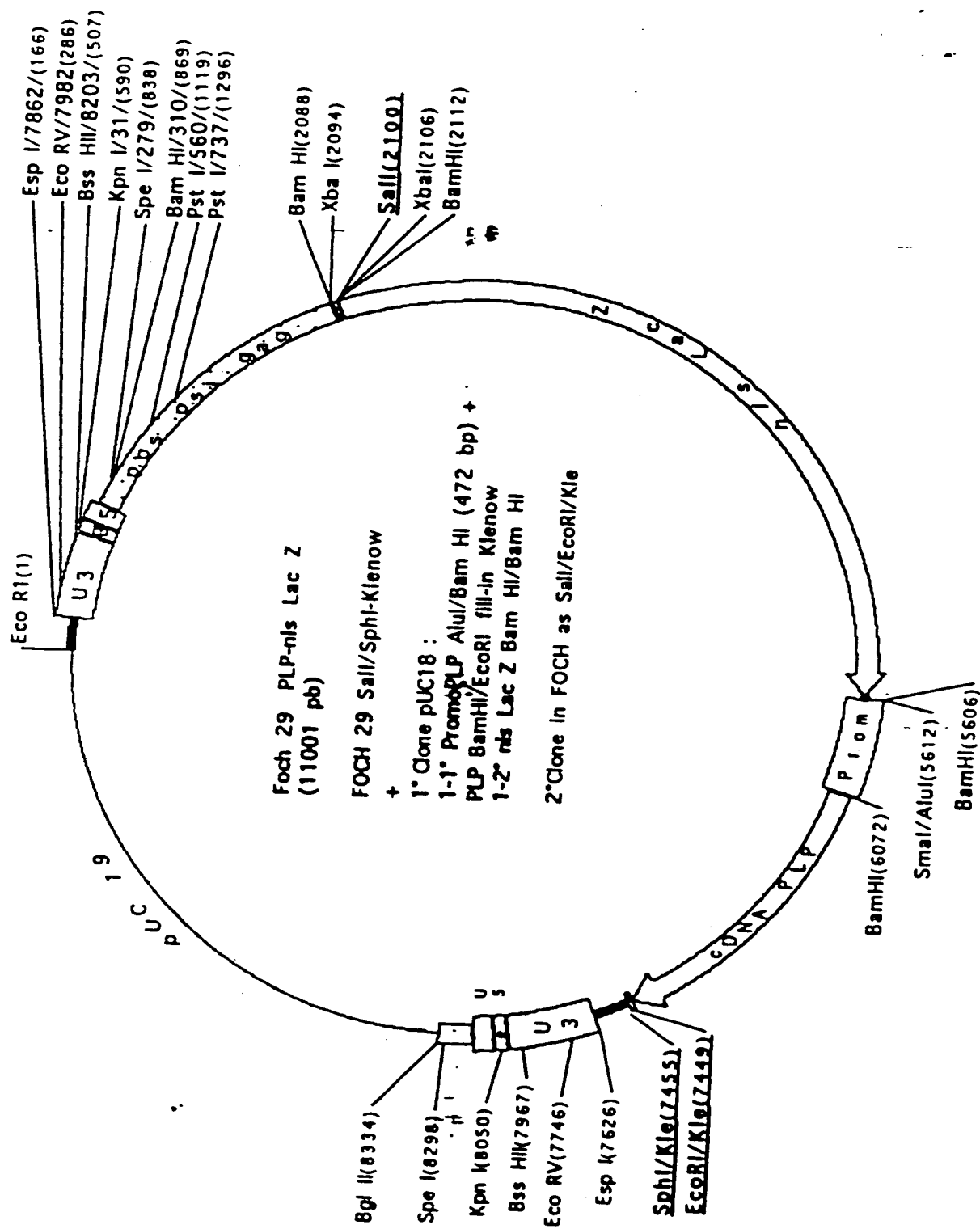
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Date of the actual completion of the international search	Date of mailing of the international search report
19 October 1994	16. 11. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl, Fax (- 31-70) 340-3016	Gurdjian, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,89 11539 (SLOAN-KETTERING INST. FOR CANCER RESEARCH) 30 November 1989 see the whole document ---	1-9, 11-14, 17-26
A	WO,A,90 06757 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 June 1990 see page 23, line 1 - line 39 -----	24

2

INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/FR 94/00806

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8911539	30-11-89	AU-B-	631554	03-12-92
		AU-A-	3740489	12-12-89
		EP-A-	0371119	06-06-90
		JP-T-	2504344	13-12-90
WO-A-9006757	28-06-90	US-A-	5082670	21-01-92
		CA-A-	2005567	15-06-90
		EP-A-	0449948	09-10-91
		JP-T-	4503351	18-06-92

A. CLASSEMENT DE L'OBJET DE LA DEMANDE

CIB 6   C12N15/86   C12N5/10   C12N5/16   C12N5/22

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 6   C12N

Documentation consultée autre que la documentation minimale dans la mesure ou ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
Y	Genbank database ,Bethesda, US GenBank ACC. NO. (GBN): Z22761 Bestwick,R. et al. Retroviral expression vector... & PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.85, no.15, 1988, WASHINGTON US pages 5404 - 5408 BESTWICK R.K. ET AL. voir le document en entier ---	1-9, 11-14, 17-26
Y	Genbank Databank Bethesda GenBank ACC. NO. (GBN): M60088 Yu,X.-F et al. Synthetic plasmid p806 polylinker site. & J.Virol. vol.64, 1990 pages 5688-5693 --- -/--	1-5

[X] Voir la suite du cadre C pour la fin de la liste des documents

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NL - 2280 HV Rijswijk
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Gurdjian, D |

Formulaire PCT-ISA 210 (deuxième feuille) (juillet 1992)

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Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
Y	WO,A,89 11539 (SLOAN-KETTERING INST. FOR CANCER RESEARCH) 30 Novembre 1989 voir le document en entier ---	1-9, 11-14, 17-26
A	WO,A,90 06757 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 Juin 1990 voir page 23, ligne 1 - ligne 39 -----	24

C.(nute) DOCUMENTS CONSIDERES COMME PERTINENTS

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)		Date de publication
WO-A-8911539	30-11-89	AU-B-	631554	03-12-92
		AU-A-	3740489	12-12-89
		EP-A-	0371119	06-06-90
		JP-T-	2504344	13-12-90
WO-A-9006757	28-06-90	US-A-	5082670	21-01-92
		CA-A-	2005567	15-06-90
		EP-A-	0449948	09-10-91
		JP-T-	4503351	18-06-92

AUSTRALIA

Patents Act 1990

NOTICE OF ENTITLEMENT
(To be filed before acceptance)

I/We, ODILE COHEN-HAGUENAUER ....

.... ACN/ARBN ....

of 35, rue Cortambert, F-75116 Paris, France ....

....

being the applicant in respect of Application No. 71893/94 ...., state the following:-

Part 1 - *Must be completed FOR ALL APPLICATIONS.*

The person(s) nominated for the grant of the patent:

    *is / ~~are~~ the actual inventor(s)

    or

    ~~*has entitlement from the actual inventor(s)~~ ....

    ....

    ~~(eg by assignment, by mesne assignment, as legal representative of ...., etc)~~

~~*Part 2* - *Must be completed IF THE APPLICATION IS ASSOCIATED with one or more*~~ PROVISIONAL APPLICATIONS.

The person (s) nominated for the grant of the patent:

    *is / *are the applicant(s) of the provisional application(s) listed on the patent request form

    or

    has entitlement to make a request under Section 113 in relation to the provisional application(s) listed on

    the patent request form ....

    ....

    (eg by assignment, by agreement, etc)

Part 3 - *Must be completed for ALL CONVENTION APPLICATIONS.*

The person(s) nominated for the grant of the patent:

    *is / *are the applicant(s) of the basic application(s) listed on the patent request form

    or

    *has entitlement from the applicant(s) of the basic application(s) listed on the patent request form

    ....

    ....

    (eg by assignment, by mesne assignment, by consent, etc)

The person(s) nominated for the grant of the patent:

  *is / *are the depositor(s) of the deposit(s) as listed hereafter

  or

  *has entitlement from the depositor(s) of the deposit(s) as listed hereafter ....

  ....

  (eg by assignment, by consent, etc)
  Deposit List (by number, deposit institution, date)

  ....
  ....
  ....

Part 7 - Must be completed if the applicant for a PATENT OF ADDITION is not the applicant or patentee of the main invention.

  I, ... ,

• the *applicant / *patentee for *application / *patent No. .... ,

  authorise ....

  to apply for a further patent for an improvement in, or modification of, the main invention.

  ....
  (Signature)   **(Date)**

• *Note: This part must be signed by the applicant/patentee of the main invention.*

Signed on behalf of
the applicant by his
Patent Attorney,   *14-2-95*
FISHER & KELLY
....
(Signature)   **(Date)**
(If the applicant is a Company or other legal entity, also indicate the name and standing of the authorized signatory.)

Note: This MUST be signed FOR ALL APPLICATIONS

* *Omit/Delete if not appropriate*